MOLECULAR MECHANISMS REGULATING THE E2F4 TRANSCRIPTION FACTOR

By

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Abstract

The E2F and Dp protein families are structurally related transcription factors that bind to specific consensus DNA sites as heterodimers. They control expression of genes that are required for cellular division to occur in a timely and ordered fashion. The six E2F proteins are regulated on many different levels. One level of regulation is the inhibition of their transactivation potential by their interaction with the retinoblastoma (Rb) family of proteins. The Rb family are in turn controlled by cyclin dependent kinases (cdks) and their regulatory subunits the cyclins and two classes of inhibitors. This thesis examines the cyclin dependent kinase control of E2F4 in an Rb family independent and dependent manner.

The direct interaction of D-type cyclins with E2F4 *in vitro* and *in vivo* is demonstrated. The interaction corresponds with phosphorylation of E2F4 both *in vivo* and *in vitro* in the presence of cdk4. The cdk4 kinase activity promotes the disruption of the E2F4/Dp-1 complex with DNA. A catalytic event is required as a kinase inactive version of cdk4 is unable to duplicate the finding. Also, cyclin E/cdk2 is unable to disrupt the DNA binding complex suggesting a specific cdk catalytic event. This role for cdk4 is corroborated with two different approaches. First, transient transfection assays demonstrate that cyclin D1/cdk4 can repress the E2F4 transactivation potential. Second, Dp-1 is unable to interact with certain phosphorylated forms of E2F4, as revealed by phosphopeptide mapping experiments. There are two domains on E2F4 that can interact with cyclin D1. The C-terminal binding site is necessary for cyclin D1/cdk4 to affect the E2F4 DNA binding potential. Taken together these studies suggest a novel regulation of E2F4 by cyclin D1 associated kinase cdk4 relating a potential requirement for some gene promoters to eliminate E2F4 DNA binding.

The role of cdk activity on E2F4 function also was investigated in a p130 dependent manner. p130, a member of the Rb family, is known to complex with E2F4 and inhibit its transactivation potential at early times during the cell cycle. As a first step to analyze how p130 causes this effect, the E2F4 binding site on p130 was mapped to a region known as the B domain.

D-type cyclin interactions with p130 occur through sequences that are distinct from sequences mediating interactions between p130 and cyclins A and E. This suggests differences in the functional outcome of p130 targeted phosphorylation. These differences may be important to the regulation of E2F4 that is complexed with p130. In this regard, the potential role of cdk2 versus cdk4 phosphorylation on the p130-E2F4 complex was examined. Cdk4 phosphorylation of p130 induces the disruption of the p130-E2F4 complex. As a means to further investigate how this occurs, the cyclin D binding site was mapped to a region of p130 that partially overlaps with the E2F4 binding site. The interaction of cyclin D1 with the pocket proteins does not rely on the Leu-x-Cys-x-Glu peptide motif in contrast to what has been suggested by others. The cdk2 regulation of E2F4 in the presence of p130 is different for cdk4. Cdk2 was unable to disrupt the p130-E2F4 complex either *in vivo* or *in vitro*. Cdk2 activity could phosphorylate E2F4 in the presence of p130. E2F4 was not phosphorylated by cdk2 in the presence of a mutant of p130 that is unable to interact with cyclins A and E, and cdk2. In addition a mutant of E2F4 that does not interact with p130 could not be phosphorylated by cdk2.

These studies suggest that cyclin D1/cdk4 disrupts p130/E2F4 interactions and E2F4/DNA interactions thereby contributing to the cell cycle control of E2F4 regulated genes.

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Dedication

This thesis is dedicated in loving memory of my father Filippo Scimè who taught me that man's greatest failure is his aspiration for mediocrity. He would have been amused.

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List of Abbreviations

γ	gamma
ATP	adenosine 5' triphosphate
cDNA	complementary DNA
CT	carboxyl terminus
cys	cysteine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
GST	glutathione S-transferase
Ip	immunoprecipitation
HEPES	4-(2-hydroxyethyl)-1-piperazineeethanesulphonic acid
kDa	kilodaltons
mRNA	messenger ribonucleic acid
M	molar
met	methionine
MOI	multiplicity of infection
NT	amino terminus
nt	nucleotides

O.D.	optical density
orf	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPO	diphenyloxazole
RAM	rabbit anti-mouse antibody
re-Ip	re-immunoprecipitation
RNA	ribonucleic acid
RPM	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris	hydroxymethyl aminomethane

Chapter 1. Introduction

1.1 The Cell Cycle and E2F

The cell cycle is the process of cell division, composed of a series of coordinated events required to replicate DNA and divide the cell to create two daughter cells. The eukaryotic cell cycle is separated into phases or stages where the prime events of DNA replication (S-phase) and cellular division (M-phase) are interspersed by growth and reorganization intervals known as Gap phases called G1 and G2 (Fig. 1.1). The G1 phase is a growth interval characterized by an increase in cell size required to prepare for DNA replication. During G1 the cell is under the influence of its environment. If the environmental conditions for division are not present the cell can arrest. S-phase is the stage where DNA replication and histone synthesis occurs. It is followed by G2 when the components required for the next stage, M-phase, are produced. The M-phase is the stage at which DNA material is equally divided between two daughter cells containing equal amounts of cytoplasm. With the completion of M-phase the cycle is brought back to G1 or if under the influence of other signals to G0. G0 is a quiescent state where cells are not actively preparing for division as in G1. Division is possible from G0 if the environmental signals for division are optimum.

Cellular division is a tightly regulated process calling upon a vast array of specialized molecules working together to bring about an ordered and controlled sequence of events. This is accomplished by regulated timing of expression and function of certain factors. One of the more important molecules involved in the cell cycle regulation is a family of transcription factors known as E2F (Dyson, 1998; Helin, 1998; Johnson and Schneider-Broussard, 1998; Nevins, 1998). The control of E2F is managed on many different levels, by a large group of proteins, including the retinoblastoma family or pocket

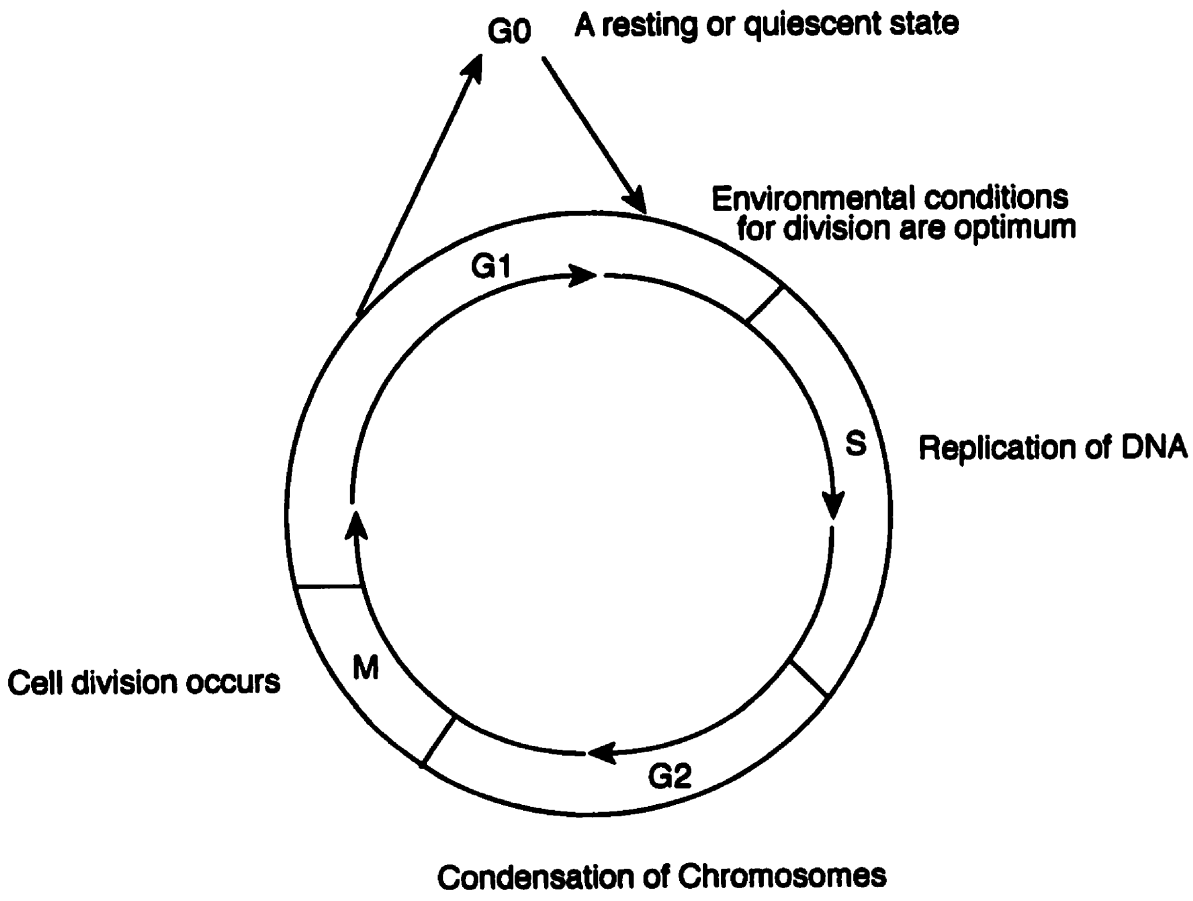

Figure 1.1 **The cell cycle.** The cell cycle is depicted with discrete stages labeled.

protein family, cyclins, cyclin dependent kinases (cdks) and cdk inhibitors (ckis). In cancer cells, a hallmark of inappropriate cell division is the dysregulation of E2F controlled gene expression.

1.2 Discovery of the E2F transcriptional activity

Isolation of the E2F transcriptional activity began with studies involving the adenovirus E2 promoter. The E2 region represents the last of the early transcriptional units to be expressed after adenovirus infection following the expression of E1A, E4, E3 and E1B (Nevins, 1987). In 1986, Kovesdi et al, employing electrophoretic mobility shift assays (EMSA), found that a factor from HeLa cell extracts bound DNA sequences in the promoter region of the E2 transcriptional unit (Kovesdi et al., 1986). The concentration of the factor increased in extracts of adenovirus infected cells. The cellular factor was termed E2F for E2 promoter binding factor (Kovesdi et al., 1987; Yee et al., 1987). Independent studies by La Thangue and Rigby characterized what turned out to be the same binding activity on the E2 promoter, from undifferentiated F9 murine embryonal carcinoma cell extracts, that was barely evident in differentiated cells, designated as differentiation-regulated transcription factor 1 (DRTF1) (La Thangue and Rigby, 1987). The activity designated as E2F/DRTF1 is actually a heterodimer made up of the closely related E2F and Dp protein family members (Bandara et al., 1993; Helin et al., 1993). The binding sites on the E2 promoter are represented by two inverted repeats of the E2F recognition motif TTTCGCGC (Loeken and Brady, 1989). Subsequent studies have revealed that the two E2F binding sites present in the E2 promoter contain DNA-protein complexes made up of the E2F1/Dp-1 heterodimer that are connected by a homodimer consisting of the 19 kdal E4 6/7 orf protein (Bandara et al., 1994; Cress and Nevins, 1994; Helin and Harlow, 1994).

1.3 Molecular Cloning of the E2F and Dp family members

There are six known E2F proteins designated E2F1 through E2F6, all capable of binding directly to the E2F DNA consensus site. E2F1 was the first E2F family member to be cloned based on its ability to bind to pRb (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). The newly cloned E2F1 molecule was confirmed by demonstrating that it could transactivate a promoter containing E2F DNA consensus binding sites. E2F2 and E2F3 were isolated by low stringency screening of cDNA libraries with an E2F1 cDNA probe (Ivey-Hoyle et al., 1993; Lees et al., 1993).

Additional E2F molecules were suggested by the inability of E2F1, E2F2 and E2F3 antibodies to completely offset abundant E2F activity present in EMSA and the presence of the retinoblastoma family members p107 and p130 in E2F-DNA complexes despite their inability to bind E2F1, E2F2 and E2F3 (Chittenden et al., 1993; Wu et al., 1995). Subsequent searches for E2F family members provided E2F4 and E2F5. E2F4 was isolated by the screening of a cDNA expression library with p107 and by PCR utilizing degenerate oligonucleotides based on E2F1 (Beijersbergen et al., 1994; Ginsberg et al., 1994). E2F5 was cloned by employing p107, p130 or Dp-1 as bait in yeast two-hybrid screening (Buck et al., 1995; Hijmans et al., 1995; Sardet et al., 1995). E2F6 was discovered by employing the Dp-1 dimerization domain as bait in yeast two-hybrid screens and from sequences obtained from the human genome project (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). Two Dp genes have been isolated thus far. Dp-1 was cloned by purifying DRTF1 from F9 cell extracts and microsequencing various peptide fragments generated from protease treatment. From the garnered amino acid data, degenerate primers for PCR were used to screen an F9 cDNA library (Girling et al., 1993). The encoding gene was designated Dp-1 for DRTF1 protein 1. Dp-2 was cloned by using the mouse Dp-1 cDNA as a probe to screen a human cDNA

Figure 1.2 Schematic representation of E2F and Dp protein families. The numbers above each protein represent amino acid positions. The functional and structural domains are highlighted.

library (Wu et al., 1995; Zhang and Chellapan, 1995). Dp-2 contains at least five splice variants encoding 3 different proteins (Ormondroyd et al., 1995; Rogers et al., 1996; Wu et al., 1995; Zhang and Chellapan, 1995).

1.4 Structural and functional analysis of E2F and Dp proteins

The E2F and Dp proteins range in size from 281 to 465 amino acids containing regions encoding functional and structural domains (Fig. 1.2). Crystallographic data for the E2F4/Dp-2 complex revealed that the region necessary for DNA binding is composed of an N-terminal winged-helix protein structure (Zheng et al., 1999). This motif consists of three α-helices and a β-sheet contributing to a hydrophobic core. The winged-helix motif is found in several eukaryotic transcription factors including HNF-3γ, HSF and ETS1 (Clark et al., 1993; Harrison et al., 1994; Kodandapani et al., 1996). The E2Fs interact with the DNA consensus sequence TTTSGCGCS (S=C or G) (Lee et al., 1998). The DNA binding domains among the E2F family are approximately 54% identical, whereas only about 40% identity exists with the Dp proteins which do not bind to DNA in the absence of E2F (Girling et al., 1993; Morkel et al., 1997). The Dp proteins are about 90% identical to each other in this domain (Morkel et al., 1997; Wu et al., 1995).

The DNA binding domain of E2Fs on its own is not efficient for proper binding. The DNA binding domain for E2F1 binds weakly to DNA and Dp-1 does not bind at all, but in the presence of both Dp-1 and E2F1 DNA binding domains the complex formation with DNA is enhanced (Bandara et al., 1993; Krek et al., 1993). The heterodimerization between E2F and Dp proteins is accomplished via a region containing a leucine-zipper motif that is present adjacent and C-terminal to the DNA binding domain found in all the E2F and Dp family members. The heterodimerization region is about 44% identical between the E2F family and 75% between the Dp family (Wu et al., 1995). E2Fs can form

homodimers but in the presence of DNA prefer to bind as heterodimers (Bandara et al., 1993; Helin et al., 1993; Ivey-Hoyle et al., 1993; Zheng et al., 1999). The Dp family members can heterodimerize with any of the E2F family members (Beijersbergen et al., 1994; Buck et al., 1995; Cartwright et al., 1998; Gaubatz et al., 1998; Hijmans et al., 1995; Sardet et al., 1995; Trimarchi et al., 1998; Wu et al., 1995). Dp proteins function by increasing the DNA binding efficiency and activation potential for the transactivating E2F family members (Bandara et al., 1993; Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Helin et al., 1993; Hijmans et al., 1995; Krek et al., 1993; Sardet et al., 1995; Verona et al., 1997). It is hypothesized that heterodimerization is essential to form an extensive protein-protein interface whose structural features are necessary to bind DNA (Zheng et al., 1999). Members of the heterodimer make similar contacts to the DNA bases in the symmetric-like portion of the DNA binding site SGCGCS. Whereas, the asymmetric portion of the site, TTT, interacts with E2Fs at an invariant amino acid motif that is missing in Dp proteins.

E2F1 through 5 contain an acidic carboxy-terminal region that corresponds to the transactivation domain and the sequences necessary to associate with the pocket proteins pRb, p107 and p130. It is not clear whether the transactivation domain can be separated from the pocket protein binding domain. A fusion protein comprised of the C-terminal 368-437 amino acids of E2F1 joined to the GAL4 DNA binding domain allowed transactivation of a GAL4 promoter construct (Kaelin et al., 1992). Discrepancies arise when a similar fusion missing 417-437 amino acids at the C-terminus, corresponding to the pRb binding domain, abolished transcriptional activity in some reports but had no effect in others (Cress et al., 1993; Helin et al., 1993; Kaelin et al., 1992; Shan et al., 1992).

The distribution of pocket protein binding with the E2F family members is not the same. E2F6 does not contain a transactivation domain nor a region to bind the pocket proteins. E2F6 acts as a transcriptional repressor by binding E2F sites at promoters

primarily containing the consensus DNA sequence TTTCCCGC (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). E2F1 through to E2F5 can associate with pRb (Helin et al., 1992; Ikeda et al., 1996; Ivey-Hoyle et al., 1993; Lees et al., 1993; Moberg et al., 1996). p107 and p130 preferentially bind to E2F4 and E2F5 (Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). The Dp proteins do not contain a transactivation domain, but they possess an acid rich carboxy terminus with 44% identity and 70% similarity that might facilitate the binding with the pocket proteins (Zhang and Chellapan, 1995). In this regard, *in vitro* translated Dp-1 has been found to interact with a pRb-GST fusion protein (Bandara et al., 1994).

Downstream of the heterodimerization domain for the E2F family lies a stretch of strong homology between the E2F molecules and absent from the Dp molecules termed the Marked box. To date no function has been ascribed to this site. Reports suggest that this region in E2F1 might be involved in E2F mediated DNA bending or provide a target site for phosphorylation resulting in the disruption of the pRB-E2F1 interaction (Fagan et al., 1994; Huber et al., 1994).

E2F1 and by homology E2F2, E2F3 and E2F6 contain a region, at the N-terminal portion, that interacts with cyclin A (Krek et al., 1994; Krek et al., 1995; Xu et al., 1994). The cyclin binding domain is not present on E2F4, E2F5 and the Dp proteins, but is similar to a conserved cyclin binding domain that is present on various proteins such as the cdk inhibitors p21, p27 and the pocket proteins (Adams et al., 1996). Cyclin A binding targets the E2F molecules and Dp-1 for phosphorylation by cdk2 disrupting the DNA binding potential (Krek et al., 1994; Krek et al., 1995; Xu et al., 1994).

E2F4 contains a stretch of repeat serine residues between the Marked box and the pocket protein binding site that is not present on the other E2Fs. The CAG oligoserines in humans is of variable size making the E2F4 gene polymorphic (Ginsberg et al., 1994). Of

55 individuals tested for E2F4 all had at least one common allele of 13 repeats. The E2F4 trinucleotide repeat sequences represent a microsatellite which are repeating oligonucleotide tracts throughout the genome. Abnormalities of such repeats, microsatellite instability (MI), have been implicated in the formation of various genetic disorders including colorectal cancers with replication/repair error phenotypes (RER) (Markowitz et al., 1995). E2F4 might be a target for mutation necessary for tumour formation. Recent reports describe the presence of variable oligoserine repeats for E2F4 in RER positive primary colorectal cancers and gastrointestinal tumours as compared to non-cancerous tissue from the same individual (Souza, 1997; Yoshitaka et al., 1996).

Based on structural and functional considerations the E2F molecules have been subdivided into three different groups. E2F1, E2F2, and E2F3 bind to pRb (Helin et al., 1992; Ivey-Hoyle et al., 1993; Lees et al., 1993), are predominantly nuclear (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997) and contain a cyclin A binding motif (Krek et al., 1994; Xu et al., 1994). Another group, consisting of E2F4 and E2F5, can also bind to pRb as well as to p107 and p130 (Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Hijmans et al., 1995; Ikeda et al., 1996; Moberg et al., 1996; Sardet et al., 1995). However, they do not contain an N-terminal extension with a cyclin A binding motif and are mainly localized in the cytoplasm (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). The recently cloned E2F6 protein represents a third type of E2F lacking a transactivation domain but retaining Dp and DNA binding capabilities (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). E2F6 is thought to function as a repressor to transcription. It does not bind to the pocket proteins but contains a putative cyclin A binding site.

1.5 The E2F responsive genes

The E2F responsive genes, implicated in the control of cellular proliferation, are expressed in a cell cycle dependent manner. Studies on E2F dependent regulation of transcription rely almost entirely on findings derived from tissue culture cells, analyzing potential E2F binding sites on relatively short pieces of genomic DNA. The number of E2F binding sites on target genes varies from one to six and they are usually located approximately 200 base pairs upstream of the start of transcription (Helin, 1998). The E2F regulated genes can be grouped into two types. One targeted group are genes involved in cell cycle control such as cyclin E (Botz et al., 1996; Ohtani et al., 1995), cyclin D (Watanabe et al., 1998), cdc2 (Tommasi and Pfeifer, 1995), p107 (Zhu et al., 1995), pRb (Shan et al., 1994), E2F1 (Hsiao et al., 1994; Johnson et al., 1994), E2F2 (Sears et al., 1997), cdc25A (Iavarone and Massague, 1999), c-Myc (Oswald et al., 1994; Roussel et al., 1994; Thalmeier et al., 1989), N-Myc (Hiebert et al., 1991), B-Myb (Zwicker et al., 1996), $p19^{ARF}$ (Bates et al., 1998) and p21 (Hiyama et al., 1998). The second group consists of genes that are essential for DNA synthesis and replication such as dihydrofolate reductase (DHFR) (Fry et al., 1997; Wells et al., 1997), DNA polymerase α (Pearson et al., 1991), thymidine kinase (Karlseder et al., 1996; Tommasi and Pfeifer, 1997), HsOCR1(Ohtani et al., 1996), cdc6 (Yan et al., 1998), histone H2A (Oswald et al., 1996), and RanBP1 (Di Fiore et al., 1999).

1.6 E2F can activate transcription and drive cells into S-phase

E2F binding is necessary to activate transcription from certain promoter sites. Mutations of the E2F binding sites in the promoter of p107, DHFR and TK lead to the inactivation of gene expression (Karlseder et al., 1996; Wells et al., 1997; Zhu et al., 1995). Transient transfection assays with E2F-reporters have shown that E2F1 through 5

can activate transcription (Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Helin et al., 1992; Hijmans et al., 1995; Shan and Lee, 1994). Furthermore, the transactivation domain of E2F transferred to the DNA binding region of GAL4 is sufficient to transactivate gene expression of a GAL4 reporter (Buck et al., 1995; Cress et al., 1993; Helin et al., 1993; Hijmans et al., 1995; Kaelin et al., 1992; Shan et al., 1992).

Overexpression of E2F using inducible systems or microinjection of E2F1 and E2F4 can drive serum starved quiescent cells into S-phase (Beijersbergen et al., 1994; Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994). S-phase entry requires intact E2F1 DNA binding and transactivation domains (Johnson et al., 1993; Shan and Lee, 1994). E2F1 has also been shown to override G1 arrest induced by TGFβ in mink lung epithelial cells (Schwarz et al., 1995). Further evidence for the E2F requirement to drive the cell cycle is demonstrated by the use of E2F1 and Dp-1 mutants that act as dominant negatives to block cell cycle progression (Hiebert et al., 1995; Wu et al., 1996). These altered proteins are deficient in DNA complex formation, but retain the ability to heterodimerize, block the cell cycle in G1 when they are overexpressed. This suggests that E2F promoted gene expression is required for cellular division.

In keeping with the notion that E2Fs control cellular proliferation is the ability of E2F1 through 4 and Dp-1 to transform immortalized cells, or in collaboration with activated Ras, primary fibroblasts (Beijersbergen et al., 1994; Ginsberg et al., 1994; Johnson et al., 1994; Jooss et al., 1995; Singh et al., 1994; Xu et al., 1995). This ability is indicated by a number of criteria such as growth on soft agar and low serum, loss of contact inhibition and the potential to induce tumours in nude mice. Experiments using the E2F1 DNA binding and transactivation domains suggest its oncogenic potential is linked to its function as a transcription factor (Singh et al., 1994; Xu et al., 1995). The ability of E2F1 to cause proliferative cellular growth is demonstrated in transgenic mice models. Transgenic mice

with E2F1 under the control of an inducible promoter develop hyperplasias and together with a Ras transgene induce skin tumourigenesis. To date there have been no reports on the amplification or overexpression of the E2F family members in human tumours. Overexpression is documented in one report in the human erythroleukemia (HEL) cell line (Saito et al., 1995).

The role of E2F in the induction of S-phase has been demonstrated by experiments performed with Drosophila embryonic cells. The mammalian analysis of S-phase induction by E2F is clouded by the large E2F family that might be functionally redundant at certain promoters. In Drosophila, a single E2F (dE2F) gene and a single Dp (dDp) gene have been identified, to date (Dynlacht et al., 1994; Ohtani and Nevins, 1994). For Drosophila cells at later stages in embryonic development dE2F is necessary for S-phase to occur (Duronio et al., 1995). dE2F is required for the expression of cyclin E, a necessary factor for the S-phase transition (Duronio and O'Farrell, 1995; Knoblich et al., 1994).

E2F transcriptional activity is the target of multiple signaling cascades. The ability of dominant negative Ras to inhibit cellular proliferation by blocking the expression of E2F responsive genes is overcome by the addition of E2F1/Dp-1 or E2F2 (Leone et al., 1997; Peeper et al., 1997). Other data points to the opposite effects of the two non-mitogenic signal transduction cascades involving the MAP kinases JNK1 and p38 on E2F activity. JNK1 is demonstrated to repress E2F activity whereas p38 promotes its activation (Wang et al., 1999).

1.7 Mechanism of E2F transcriptional activation

Three different mechanisms have been proposed for how E2F activates transcription. A simple hypothesis relates the binding of E2F to the basal transcriptional apparatus via the TATA Binding Protein (TBP) to induce gene transcription (Hagemeier et al., 1993). Another mechanism suggests that transcription is stimulated by the recruitment

of co-factors. The transcriptional co-activator CBP/p300 has been demonstrated to bind to the transactivation domain of E2F1 (Trouche and Kouzarides, 1996). p300/CBP possesses histone acetylase activity that can disrupt nucleosome structures thereby altering chromatin and affecting transcription. Another co-factor TRRAP (transformation/transcription domain-associated protein) that is essential for the c-Myc transformation of mammalian cells has been shown to bind to the transactivation domain of E2F1. TRAPP might be a universal co-factor essential for the transcription of genes required for proliferation as it interacts with the transformation domain of c-Myc (McMahon et al., 1998). A third proposal relies on the potential of E2F to bend DNA (Cress and Nevins, 1996). The end effect of this is to alter the DNA architecture making it more likely for other promoter bound transcription factors, such as Sp-1, to come in contact with the basal transcriptional machinery (Karlseder et al., 1996). The above described mechanisms are not necessarily mutually exclusive.

1.8 E2F can repress gene expression

E2F can also act as a transcriptional repressor when bound to certain promoter sites. Mutation of the E2F consensus sequence in the promoters for cyclin E, p21, E2F1, E2F2, cdc25A and HsORC1 results in the enhancement of their mRNA expression level (Iavarone and Massague, 1999; Johnson, 1995; Johnson et al., 1994; Ohtani et al., 1996; Ohtani et al., 1995; Sears et al., 1997). Evidence from *in vivo* footprinting experiments on the b-myb and cdc2 promoters suggests that repression might occur in the G0/G1 phase of the cell cycle when the promoter sites are bound by E2F4 (Tommasi and Pfeifer, 1995; Zwicker et al., 1996). At later stages of the cell cycle when the genes for these promoters are transcribed there is no E2F binding activity. These results suggest that gene expression ensues with the loss of E2F complex formation. The suppressive role for E2F is made possible by the binding of pocket proteins and is discussed in detail below.

1.9 Transcriptional regulation of E2F

Transcriptional control is one method by which E2Fs are regulated. Studies involving the measurement of protein or mRNA levels in quiescent fibroblasts or resting T-cells stimulated to enter the cell cycle demonstrate that the expression pattern for the E2F family members is different. The mRNA levels for E2F1 and E2F2 are significantly increased in the G1/S phase of the cell cycle (Hsiao et al., 1994; Johnson et al., 1994; Kaelin et al., 1992; Sears et al., 1997). In fact, E2F1 and E2F2 contain E2F promoter sites which might act in an auto regulatory fashion and/or be occupied by other E2Fs (Hsiao et al., 1994; Johnson et al., 1994; Sears et al., 1997). The transcriptional profile for E2F3 is slightly different at least in REF52 cells. E2F3 is expressed beginning in the G1/S phase when G0 cells are growth stimulated. Its expression continues to cycle with successive cell divisions, whereas E2F1 and E2F2 do not reappear after the initial G1/S transition from quiescence (Leone et al., 1998). E2F5 mRNA levels are increased very early in G1 fibroblasts with levels rising 12 fold as compared to E2F4 which undergoes a 2-3 fold increase (Sardet et al., 1995). E2F4 and Dp-1 are constitutively expressed including during G0 with E2F4 representing the most actively transcribed member of the family (Lindeman et al., 1997; Moberg et al., 1996; Muller et al., 1997; Vairo et al., 1995; Verona et al., 1997). The E2F family gene expression pattern suggests that E2F1, 2, and 3 might be important in cell growth, whereas E2F4 and Dp-1 might be involved during the quiescent phase. To date, the cell cycle expression patterns for E2F6 and Dp-2 have not been reported.

1.10 Regulation of E2F by pocket proteins

1.10.i Identification of pRb regulation of E2F by the interaction with DNA tumour viruses

Another method of E2F regulation is protein-protein interaction with the Rb family. The Rb family in turn is under the influence of cyclins, cdk's and their inhibitors cki's. The pocket proteins can form repressor complexes with the E2F family of transcription factors. The ability of the DNA tumour virus proteins, adenovirus E1a, SV40 large T-antigen and human papillomavirus (HPV) E7, to drive quiescent, terminally differentiated cells into S-phase and promote transformation provided the link between E2F and the Rb protein family (Bayley and Mymryk, 1994; Fanning, 1992; Phelps et al., 1992). Early reports had shown that E1a was required for expression of the E2 transcriptional unit based on mutational studies of the E2 promoter (Loeken and Brady, 1989). The DNA sequences that are necessary for the induced transcription contain E2F consensus binding sites. E1a, lacking a sequence specific DNA binding activity, induces transcription indirectly by promoting the activation of E2F (Chatterjee et al., 1988; Ko et al., 1986). E1a was also found to transactivate certain promoters of cellular genes related to the E2F binding site (Blake and Azizkhan, 1989; Hiebert et al., 1989; Thalmeier et al., 1989). The previous results and the finding that large T-antigen and E7 could also promote transcription from the E2 promoter suggested that the DNA tumour virus oncoproteins were influencing the activity of E2F by the same mechanism to obtain a growth advantage over the infected cell (Loeken et al., 1986; Phelps et al., 1988).

The method by which the oncoproteins regulated E2F function was unraveled by the findings that E1a could interact with a variety of cellular proteins (Harlow et al., 1986; Yee and Branton, 1985). The first identified was the interaction with a 110 kdal protein that was found to be the retinoblastoma susceptibility protein (pRB) (Whyte et al., 1988).

pRb represents the prototypic tumour suppresser protein (Weinberg, 1995). The retinoblastoma gene has been directly implicated in the formation of hereditary retinoblastoma by mutation of a second allele after inheritance of one mutated allele (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). This was first hypothesized in Knudson's rate limiting "two-hit" model that proposes the requirement of two mutations for the formation of retinoblastoma (Knudson, 1971). In addition, the mutation or loss of the pRb gene is found in many different cancer types such as, cervical carcinomas, small cell lung carcinomas, bladder carcinomas, breast carcinomas, prostate carcinoma, primary leukemias, oesteosarcomas and soft tissue sarcomas, suggesting that it is an important target for disruption leading to oncogenesis (Bookstein et al., 1990; Friend et al., 1986; Friend et al., 1987; Harbour, 1988; Horowitz et al., 1989; Mendoza et al., 1988; T'Ang et al., 1988; Toguchida et al., 1988; Weichselbaum et al., 1988). pRb's growth suppressive properties are evident by its re-introduction in prostate, breast, bladder and osteosarcoma cell lines that are deficient for it (Bookstein et al., 1988; Huang, 1988; Takahashi et al., 1991; Wang et al., 1993). The suppression of the neoplastic phenotype in these cells is exemplified by slowed growth and increased average cell size. Nude mice that were injected with the tumour cells harbouring newly functional pRb did not develop tumours as opposed to the mice that were injected with pRb negative cells. The importance of the pRb-E1a interaction to cell dysregulation is evident from mutants of E1A that are unable to transform cells do not bind to pRb (Egan et al., 1988; Jelsma et al., 1989; Whyte et al., 1988; Whyte et al., 1989). Furthermore, E7 and large T-antigen interact with pRb and their transforming ability is dependent on this interaction (DeCaprio et al., 1988; Dyson et al., 1989; Moran, 1988; Phelps et al., 1988).

It has been demonstrated that the basis by which E1a, E7 and large T-antigen interact with pRb is to disrupt the interaction with E2F. In this manner, transcriptional repression caused by pRB binding to E2F is alleviated by the DNA tumour virus

oncoproteins and expression for proliferative genes can progress. A hint that E1a caused this to happen is detailed in reports that described the E1a disruption of host cell protein complexes releasing E2F that was utilized for E2 transcription (Bagchi et al., 1990; Raychaudhuri et al., 1991). The regions of E1a that were required to dissociate this complex were also necessary to bind to pRb. Many studies have reported the ability of E1a to dislodge the interaction of pRb with E2F (Bagchi et al., 1991; Bandara and La Thangue, 1991; Cao et al., 1992; Chellappan et al., 1991; Chittenden et al., 1991; Shirodkar et al., 1992). In addition, E7 and large T can also abrogate the Rb-E2F complex revealing that the DNA tumour virus oncoproteins function by the same mechanism to regulate E2F activity (Chellappan et al., 1993). Taken together, these results suggest that the DNA tumour viruses act to drive the cell cycle and transform cells partly through the interaction with pRb to release E2F.

1.10.ii pRb-E2F complex formation functions as a negative regulator of cell proliferation

The interaction between pRb and E2F provides a model for a mechanism of pRb mediated growth regulation contributing to its tumour suppressor capability. Different lines of evidence other than the interaction with oncoproteins suggest that pRB functions as a negative regulator of cell proliferation through its interaction with E2F. For example, mutant pRb molecules found in tumourigenic cells are unable to bind E2F or they contain mutations within the domain that is required for stable interaction (Bandara and La Thangue, 1991; Chellappan et al., 1991; Cobrinik, 1996; Horowitz et al., 1990; Horowitz et al., 1989; Kaelin et al., 1991). The addition of pRb into osteosarcoma cells, lacking a functional pRb protein, blocks cells in S-phase if microinjected in early G1 or if transiently transfected (Goodrich et al., 1991; Hinds et al., 1992). This function of pRb correlates with the potential to bind E2F. The minimal region of pRb required to block the cell cycle

is represented by the E2F binding domain (Hiebert, 1993; Qian et al., 1992; Qin et al., 1992).

pRb can inhibit growth by forming a transcriptional suppressor complex with E2F on promoter sites thereby inhibiting the expression of E2F target genes. This function for pRb has been confirmed by many different experimental approaches. As an example, the capacity of pRb to repress E2F mediated transactivation is described in experiments employing transient transfection assays with simple E2F-promoter constructs (Hiebert et al., 1992; Weintraub et al., 1992; Zamanian and La Thangue, 1992). The suppression by pRB is dependent on direct binding to E2F that is bound to DNA. Mutations within the E2F binding site of the myc promoter that alleviate E2F interaction can no longer be suppressed by pRB (Hamel et al., 1992). In addition, pRb can suppress transactivation by a fusion protein containing the E2F1 transactivation domain and the GAL4 DNA binding domain. GAL4/E2F1 mutants that transactivated gene expression but did not bind to pRb were no longer suppressed by pRb overexpression. Mutants of pRb that did not bind E2F lost the ability to suppress (Flemington et al., 1993; Helin et al., 1993).

Recent data in support of E2F forming a suppresser complex with pRb are from experiments with a stably transfected E2F1 mutant that retains its DNA binding potential but is deficient in the C-terminal transactivation and pocket protein binding domains (Zhang et al., 1999). This mutant is assumed to mimic the DNA binding potential of the E2F family and is proposed to bind all the available E2F sites. The stable cell lines expressing the mutant are not blocked in the cell cycle by pRb suggesting that the transactivation potential of E2F is not necessary for cell cycle progression, but is required to form a repressor complex with pocket proteins.

1.11 p107 and p130 can also suppress E2F transactivation

p107 and p130, members of the pocket protein families named according to their apparent molecular weights in polyacrylamide gels interact with E1a (Harlow et al., 1986; Whyte et al., 1988). p107 and p130 are more alike at 50% similarity, as compared to pRb that has a similarity of 30% with p107 and p130 (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). The Rb family shares extensive homology primarily at the two disparate A and B domains which comprise the pocket domain. The pocket domain is necessary to bind the oncogenes of DNA tumour viruses containing the Leu-x-Cys-x-Glu (LxCxE) motif (Fig. 1.3)(Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). The A and B domains are separated by a small region known as the spacer element. The spacer region on pRb is small as compared to the spacer regions in p130 and p107. The spacer element is significant for it relates a functional difference between pRb and both p107 and p130 that is described below.

p107 and p130 interact with E2F4 and E2F5. p107, like pRb, requires the A and B domains of the pocket region for binding E2F. The binding domain is made up of overlapping amino acids that are required to bind DNA tumour virus oncoproteins (Hiebert et al., 1992; Qian et al., 1992; Smith and Nevins, 1995; Zhu et al., 1995). It is hypothesized that the A and B domains provide a functional interface by which E2F can bind (Lee et al., 1998). An E2F binding domain on p130 has not been deduced. In addition, a potential Dp-1 interaction with pocket proteins is described by early *in vitro* experiments for Dp-1 and pRb, but not for p107 and p130 (Bandara et al., 1994).

The pocket proteins p107 and p130 are able to inhibit the cell cycle in G1 as determined by flow cytometry analysis of transfected cell lines (Vairo et al., 1995; Zhu et al., 1995; Zhu et al., 1993). Furthermore, mutagenesis of p107 reveals that a region

Figure 1.3 The pocket protein or Rb family. The pocket proteins are shown schematically with numbers above each protein representing amino acids. A and B denote the A/B pocket domain and SP the spacer element. The binding domains for various proteins are outlined and described in the text.

mapping to the E2F binding domain can cause growth suppression (Zhu et al., 1995). The growth suppression by p107 correlates to the ability to repress E2F-mediated transactivation (Beijersbergen et al., 1994; Ginsberg et al., 1994; Schwarz et al., 1993; Smith and Nevins, 1995; Zamanian and La Thangue, 1993; Zhu et al., 1993).

The evidence that p107 and p130 are true tumour suppressers is not as strong as for pRb. Mutations in primary cancer cells for p130 and p107 have not yet been discovered. To date, a small cell lung carcinoma cell line harbouring a p130 mutation represents the only mutation for p130 that has been reported in cancer cell lines (Helin et al., 1997). Gene targeting experiments reveal contradictory findings resulting from the genetic background of the mice. Initial experiments for targeted p107 or p130 disruption revealed no phenotypic abnormalities in mice with the genetic background 129/Sv:C57BL/6J (Cobrinik et al., 1996; Lee et al., 1996). However, when both genes were targeted simultaneously mice had defects in chondrocyte development suggesting that p107 and p130 are functionally redundant. These results contrast with recent reports for the knockout experiments performed in a BALB/cj genetic background that suggest a suppressive cellular proliferative role for p107 (LeCouter et al., 1998). p107-/- animals exhibit myeloid hyperplasia in the spleen and liver. In addition, chimeric mice generated with $Rb^{-/-}/p107^{-/-}$ embryonic stem cells develop retinoblastoma, if the loss of both pRb and p107 occurs in the retina (Robanus-Maandag et al., 1998).

1.12 Mechanism of Pocket Protein repression

The mechanism of repression by pocket proteins is not completely understood. The simplest explanation is that the pocket proteins bind to a region in the E2F transactivation domain blocking its ability to interact with the basal transcriptional apparatus (Hagemeier et al., 1993; Shao et al., 1997; Siegert and Robbins, 1999; Weintraub et al., 1992). Another hypothesis suggests that the pocket proteins actively repress transcription in a repressor

complex with E2F. In this regard, E2F is not involved in transactivation but is required to bind DNA to tether the pocket proteins to the promoter region whereupon active transcriptional repression of adjacent enhancer sites is made possible. The GAL4 DNA binding domain fused to pRB or p107 can repress transcriptional activity of a GAL4 reporter construct (Bremner et al., 1995; Chow and Dean, 1996; Sellers et al., 1995; Starostik et al., 1996; Weintraub et al., 1995). Thus, pRb or p107 brought to a promoter site in an E2F independent fashion retains the ability to repress transactivation. In support of this view are two lines of evidence. In experiments to identify pRb DNA targets, despite the potential of pRb to interact with many different transcription factors, only E2F consensus sites were identified (Chittenden et al., 1991). This suggests pRb interacts with other transcription factors and inhibits their transactivation potential in context with its potential to interact with E2F. Also, many different enhancer sites, with the ability to induce transcription, are in proximity to E2F binding sites (Karlseder et al., 1996; Lin et al., 1996; Loeken et al., 1986; Watanabe et al., 1998).

The Rb family might be actively repressing by influencing histone deacetylation by binding to histone deacetylases (HDAC1) (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Histone deacetylases promote the formation of nucleosomes that are thought to impede the positive regulatory action of transcription factors with the basal transcriptional machinery (Grunstein, 1997). HDAC1 was demonstrated to repress E2F mediated transactivation in a synergistic manner with pRB. In this manner, pRb is thought to alter chromatin structure resulting in inhibition of transcription. The recruitment of inhibitory factors might be another potential repressor mechanism, for example the binding of HBP1 to p130 and pRb (Tevosian et al., 1997). HBP1, a member of the high mobility group (HMG) class of transcription factors, is hypothesized to repress transcription of genes with promoters containing E2F binding sites.

There are additional regulatory functions on E2F ascribed to the pocket proteins other than suppression of gene expression. One function is the enhancement of E2F stability against the ubiquitin-proteasome pathway (Hateboer et al., 1996; Hofmann et al., 1996). This is accomplished by direct binding of pRb to E2F1 and p107 and p130 to E2F4. Another level of control implicates the Rb family in influencing the subcellular localization of E2F4 and E2F5. E2F4 and E2F5 are normally cytosolic and lack a nuclear localization signal but upon co-expression of p107 or p130 become overwhelmingly compartmentalized in the nucleus (de la Luna et al., 1996; Magae et al., 1996).

1.13 Regulation of pocket proteins by phosphorylation

The pocket proteins are phosphorylated in a cell cycle dependent manner. pRb is in a minimally phosphorylated (underphosphorylated) state in G1. It continually becomes phosphorylated as cells pass from G1 to S phase and at the end of mitosis it returns to an underphosphorylated state (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). p107 and p130 are also phosphorylated in a cell cycle specific manner in studies employing quiescent mouse fibroblasts or a glioblastoma cell line induced to proliferate from quiescence (Beijersbergen et al., 1995; Mayol et al., 1995; Xiao et al., 1996). Limited data is available on the dephosphorylation of pocket proteins. pRb has been shown to bind directly to the protein phosphatase 1 (PP1) catalytic subunit during mitosis (Durfee et al., 1993). The electroporation of active PP1 into G1 cells prevented S-phase entry and pRb phosphorylation (Berndt et al., 1997). Recently, a regulatory subunit, PR59, for protein phosphatase 2A (PP2A) has been shown to interact directly with p107 resulting in its dephosphorylation by recruiting the catalytic subunit of PP2A (Voorhoeve et al., 1999).

1.14 Cyclins target pocket proteins for phosphorylation by cdks

The pRb family is phosphorylated at the amino acids that fall within the consensus target sites for cdks (Lees et al., 1991; Lin et al., 1991). The cyclical activity of cdks is necessary for cellular division to occur in a timely and ordered manner (Sherr, 1996). Cdks phosphorylate the substrates needed for cell cycle progression including the pocket proteins and E2Fs. Cdks are activated by the interaction of a family of related proteins known as cyclins. Cyclins are expressed in a periodic fashion at certain points during the cell cycle. The expression pattern for each of the cyclins varies suggesting their involvement at distinct steps in the cell cycle. Thus, the activities of the catalytic subunits are controlled in a cell cycle dependent manner.

To date, at least 16 cyclins and 8 cdks have been cloned resulting in a complex array of regulatory phosphorylation. Cyclin interaction with cdks is mediated by a conserved 100-150 amino acid domain known as the cyclin box. Cyclins also contain a region of homology known as the destruction box or a PEST sequence that allows the protein levels to be turned over in a cell cycle specific manner (Glotzer et al., 1991; Rogers et al., 1986). The cyclins operate for the most part during specific phases of the cell cycle and are loosely divided into two subgroups, the G1 and G2 cyclins. The cyclins A, E, and the D family including D1, D2, and D3 represent the important cyclins involved in the direct control of the pocket protein and E2F families.

1.14.i The D cyclin family promotes G1 cell cycle progression

D-type cyclin appearance is growth factor dependent. They are the first of the G1 cyclins to be expressed. The kinetics of expression for cyclin D1 and D2 begin in early G1 and become maximal at the G1/S transition (Won et al., 1992.). The kinetics for cyclin D3 is slightly different with synthesis beginning at the G1/S border and peaking at S-phase (Motokura et al., 1992). The D-type cyclins preferentially activate cdk4 and cdk6 with the

cyclin D1/cdk4 activity becoming maximal in early G1 to the mid G1 phase. (Bates et al., 1994; Matsushime et al., 1992; Matsushime et al., 1994; Meyerson and Harlow, 1994; Tsai et al., 1991; Xiong et al., 1992).

The individual expression pattern for the D-cyclins is tissue specific and cell line specific but in a partly overlapping manner as more than one family member can be expressed in the same tissue and cell line (Bartkova et al., 1998; Lukas et al., 1995; Lukas et al., 1994). The tissue restricted expression pattern is apparent in gene targeting experiments. Cyclin D1 targeted disruption in mice results in aberrant development of the retina and mammary epithelium (Fantl et al., 1995; Sicinski et al., 1995). This contrasts with cyclin D2 disruption that results in mice defective in the growth of ovarian granulosa cells and the testes (Sicinski et al., 1996).

The cyclin D family is required for G1 progression. Disruptions of the D-type cyclins with microinjection of sequestering antibodies into different cell lines block the cell cycle in G1 (Baldin et al., 1993; Bartkova et al., 1998; Lukas et al., 1995; Lukas et al., 1994; Quelle et al., 1993). The importance to the control of early G1 events is evident from microinjection experiments. There is no block to the cell cycle if cyclin D1 antibodies are injected at a point late in G1 (Quelle et al., 1993). On the other hand, experiments that overexpress D-type cyclins can shorten the G1 interval and produce cells that are smaller than their normal counterparts (Ando et al., 1993; Herzinger and Reed, 1998; Quelle et al., 1993; Resnitzky et al., 1994).

The inappropriate expression of cyclin D1 leads to a loss of growth control. This is exemplified by the overexpression of cyclin D1 and D2 in several types of human tumours (Hunter and Pines, 1994; Motokura and Arnold, 1993). Cyclin D1 was originally identified as the PRAD1 gene (Motokura, 1991). PRAD1 overexpression is suspected as the cause of some parathyroid adenomas by rearrangement to the promoter site of the parathyroid hormone gene. In addition, cyclin D1 is a strong candidate for amplification

leading to various leukemias by translocation to the immunoglobulin heavy chain gene locus (Motokura and Arnold, 1993). Cyclin D2 is the vin-1 gene which is the site of insertional mutagenesis of murine leukemia virus induced T-cell leukemia (Hanna et al., 1993). Furthermore, cyclin D1 transgenic mice impede lymphocyte maturation and lymphogenesis is induced in collaboration with a Myc transgene (Bodrug et al., 1994; Lovec et al., 1994). Mice expressing the cyclin D1 transgene under the control of the mouse mammary tumour virus long terminal repeat induce mammary gland hyperplasia and carcinomas (Wang et al., 1994). In tissue culture cells cyclin D1 and D2 can cooperate with activated Ha-Ras to transform primary fibroblast cells (Hinds et al., 1994; Kerkhoff and Ziff, 1995; Lovec et al., 1994).

1.14.ii Cyclin E can promote entry into S-phase

Cyclin E is also essential to the cell cycle progression in G1. Cyclin E is synthesized at a time after D-type cyclin expression at about mid G1 and peaks later in the G1/S phase (Dulic et al., 1992; Koff et al., 1991; Koff et al., 1992). Cyclin E can interact with cdk2 , but its predominant *in vivo* activity is associated with cdk2 at G1/S (Dulic et al., 1992; Koff et al., 1991; Koff et al., 1992). As with cyclin D, cellular microinjection of anti-cyclin E antibodies is able to block cells in G1 (Ohtsubo et al., 1995). If cells are microinjected at a point after the G1/S transition they are no longer blocked in proliferation suggesting cyclin E activity is not required after this point for cell cycle progression. The cyclin E control of the G1/S transition is dependent on its catalytic partner cdk2. This is apparent from the use of a dominant negative cdk2, that does not harbour kinase activity but can still bind to cyclin E. Dominant negative cdk2 is sufficient to block the cell cycle in G1 as well (van den Heuval and Harlow, 1993). Other experiments suggesting that cyclin E is essential in controlling G1 progression are from studies in which cyclin E is overexpressed by the use of an inducible promoter in mammalian fibroblastic cells.

Overexpression of cyclin E diminishes the requirement for serum, accelerates the G1 phase of the cell cycle and decreases cell size (Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995; Resnitzky et al., 1994; Wimmel et al., 1994). If cyclin D1 and cyclin E are over expressed by inducible promoters in the same cells the effect of shortening the cell cycle progression in G1 is enhanced (Resnitzky and Reed, 1995).

There are examples of alterations of cyclin E expression with tumour formation. Cyclin E amplifications have been documented in many tumour types but are most common in breast carcinomas (Keyomarsi et al., 1995; Keyomarsi et al., 1994). Transgenic mouse experiments also maintain the potential for cyclin E to be oncogenic. A cyclin E transgene under the control of a β-lactoglobulin promoter, which directs transgene expression to the mammary gland during pregnancy and lactation, exhibits the induction of mammary gland hyperplasia and carcinomas in affected animals (Bortner and Rosenberg, 1997).

1.14.iii Cyclin A is required for S-phase and the G2/M transition

Human cyclin A was isolated as an E1a associated protein and found to be the target of integration of the hepatitis B genome in hepatocellular carcinomas suggesting a possible target to allow for viral replication and growth (Giordano et al., 1989; Wang et al., 1990). Cyclin A synthesis increases at a point after cyclin E at about the onset of S-phase (Girard et al., 1991; Pines and Hunter, 1991; Pines and Hunter, 1990). Cyclin A interacts *in vivo* with either cdk1 or cdk2 (Elledge and Spottswood, 1991; Pines and Hunter, 1990; Tsai et al., 1991). Cyclin A is active at S-phase and the G2/M transition. As with the D-type cyclins and cyclin E, the microinjection of cyclin A antibodies in G1 induces a block to cell proliferation (Girard et al., 1991). However, the microinjection of anti-cyclin A antibodies in G2 translates into the inhibition of mitosis (Pagano et al., 1992). This is expected from the interaction of cdk1 that is known to be active at later stages of the cell cycle. Cyclin A

has been implicated as a target of adhesion-dependent signals that control cell proliferation. Anchorage-independent cell proliferation is a hallmark of oncogenic transformation. The ectopic expression of cyclin A induces anchorage independent growth (Guadagno et al., 1993). As will be noted later, cyclin A activity can also regulate E2F1 DNA binding potential.

1.14.iv Inhibitors of cdks

An additional level of complexity is the inhibition of cyclin/cdk activity by two classes of inhibitor molecules known as cyclin dependent kinase inhibitors (cki's). Cki's are divided into two groups based on sequence homology and their targets for inhibition. One class of inhibitors are the universal inhibitors that can target cyclin A/cdk2, cyclin E/cdk2 and cyclin D/cdk4 for inhibition. These include the proteins p21 (Cip1, Waf1, Sdi1, Cap20) (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Noda et al., 1994; Xiong et al., 1993), p27 (Kip1) (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57 (Kip2) (Lee et al., 1995; Matsuoka et al., 1995). The CIP/KIP family contain separate cyclin and cdk binding motifs that are highly conserved at their N-terminus. They interact with both the cyclin and cdk molecules to inhibit activity (Chen et al., 1995; Goubin and Ducommun, 1995; Lin et al., 1996; Luo et al., 1995; Russo et al., 1996).

The second class of inhibitors are a highly homologous family of proteins that contain at least three ankyrin repeats and are known as the INK4 family (Sherr and Roberts, 1995). The INK4 proteins specifically target D-type cyclin/cdk4 and cdk6 activity by binding to the kinase thereby competing away the cyclin interaction. This class of molecules includes p16 (INK4a, MTS1, CDKN2, CDK4I) (Serrano et al., 1993), p15 (INK4b) (Hannon and Beach, 1994), p18 (INK4c) (Guan et al., 1994; Hirai et al., 1995) and p19 (INK4d) (Hirai et al., 1995).

Most cellular anti-proliferative signals lead to the induction of cki's. For example the replicative senescence of human fibroblasts involves the induction of p16 and p21

(Alcorta et al., 1996; Brown et al., 1997; Noda et al., 1994). Quiescence due to contact inhibition entails an increase in expression of p27 (Polyak et al., 1994). Anti-mitogenic substances that block the cell cycle such as TGF-β prompt the expression of p15 (Hannon and Beach, 1994). Cell cycle checkpoints such as DNA damage induce the production of p21 by a p53 dependent mechanism (El-Deiry et al., 1993). p57 gene targeting experiments describe mice that have complex tissue overgrowth and pre-disposition to cancer consistent with a role in the Beckwith-Wiedemann syndrome (BWS) (Zhang et al., 1997). The chromosomal region involved in the formation of BWS maps within 80 kilobases of the p57 gene locus and some patients have been shown to be heterozygous for mutations in the p57 gene (Hatada et al., 1996; Hoovers et al., 1995). Finally p16 mutations are found in as many as one third of all human cancers thus exemplifying its role as a major tumour suppresser (Kamb et al., 1994; Norbori et al., 1994).

1.15 Cyclin/cdk targeting of pRb affects regulatory control of E2F

Details on the phosphorylation of pRb by the cdks and cyclins is obscured by the large number of cyclins and cdks and the number of sites that can be phosphorylated on pRB. There are 16 Ser/Thr-Pro motifs which are potential cdk phosphorylation sites with at least 11 of these shown to be phosphorylated *in vivo* (Lees et al., 1991; Lin et al., 1991). Added to this conundrum is the potential of various cyclin cdk combinations to phosphorylate pRB *in vivo* and *in vitro*.

Ectopic expression of cyclin A and cyclin E can enhance the phosphorylation of pRb. The kinase activity relates to the ability of these cyclins to overcome a pRB induced block of the cell cycle suggesting the rescue of E2F repression (Hinds et al., 1992; Horton et al., 1995). The overexpression of D-type cyclins can also result in the phosphorylation of pRb that correlates with E2F loss and cell cycle progression (Horton et al., 1995).

Unlike cyclins A and E, the D-type cyclins can form stable complexes with pRb (Dowdy et al., 1993; Ewen et al., 1993). One study suggested that cyclin D1 interacts with pRB through its amino acid motif L-x-C-x-E that is used by the DNA tumour virus oncoproteins to bind pRb (Dowdy et al., 1993). The stable binding to pRb suggests that cyclin D/cdk4 activity directed against pRb-E2F may dissociate the complex (Kato et al., 1993). The timing of pRb phosphorylation by D-type cyclins with their kinase partners cdk4 and cdk6 in mid to late G1 correlates with this finding (Ewen et al., 1993; Matsushime et al., 1992; Meyerson and Harlow, 1994).

There is strong evidence for the phosphorylation of pRb as the only *in vivo* outcome of targeted cyclin D kinase activity. A cell cycle block in G1 from the ectopic addition of the specific cdk4/cdk6 inhibitor p16 (Guan et al., 1994; Hirai et al., 1995; Lukas et al., 1995; Medema et al., 1995) or the targeted disruption of the cyclin D protein family (Baldin et al., 1993; Bartkova et al., 1998; Lukas et al., 1995; Lukas et al., 1995; Lukas et al., 1995; Quelle et al., 1993) requires a functional pRb protein.

It is not known whether cyclin D/cdk4 or cdk6 activity is sufficient to cause the disruption of the pRb-E2F complex on its own. The potential for pRb to be phosphorylated *in vitro* at cdk2 and cdk4 distinctive sites reveals possible mechanistic models for how pRb might be controlled by phosphate addition (Adams et al., 1999; Connell-Crowley et al., 1997; Kitagawa et al., 1996; Zarkowska and Mittnacht, 1997). One interpretation is that cyclin/cdk complexes may be able to exert distinct effects on pRb function other than the regulation of E2F. Phosphorylation of specific sites may remove the ability of the DNA tumour viruses to bind, but not E2F and vice versa [(Knudsen and Wang, 1996; Knudsen and Wang, 1997). In addition, different cyclin/cdk combinations might be able to phosphorylate pRB with the same outcome. For example, cyclin E or D activity on their own is sufficient to release bound E2F (Ewen et al., 1993; Hinds et al., 1992; Horton et al., 1995). Another hypothesis has cyclins cooperating to bring about the

same outcome. Recent reports demonstrate that pRb must be phosphorylated by cyclin D/cdk4 followed by cyclin E/cdk2 to cause the disruption of complex formation with E2F and that no one cdk binding site regulates the interaction (Brown et al., 1999; Lundberg and Weinberg, 1998).

1.15.i Cyclin/cdk targeting of p107 and p130 affect the control of complex formation with E2F

The role of phosphorylation by the cdks is less defined for p107 and p130 as compared to pRb. p107 and p130 have been shown to bind cyclins A, E and the D family *in vivo* and *in vitro* (Ewen et al., 1992; Faha et al., 1992; Hannon et al., 1993; Li et al., 1993). Initial reports demonstrated that p130 and p107 can be phosphorylated *in vitro* by cyclin A, cyclin E and cyclin D1. As with pRb, conflicting data exist as to the exact cyclin kinase activity that controls the ability to interact with E2F. The effect of phosphorylation by cyclin D1 and D2 on p107 has been shown to disrupt its potential binding to E2F4 (Beijersbergen et al., 1995; Xiao et al., 1996). However, other reports studying the effects of phosphorylation on the interaction of p107 with E2F4 in electrophoretic mobility shift assays describe the potential of recombinant cyclin A/cdk2 to disrupt the complex interaction with E2F4 on DNA (Suzuki-Takahashi et al., 1995; Zhu et al., 1995). For p130, *in vitro* experiments with purified recombinant p130 with cyclin A, cdk2 and E2F4 results in the release of p130 from the DNA binding complex (Mayol et al., 1996). However, cyclin D3/cdk4 activity has been correlated with the timing of p130 phosphorylation and release of E2F4 binding in murine fibroblasts (Dong et al., 1998).

1.16 The temporal binding of E2F-pocket protein complexes

The pocket proteins show different temporal and structural patterns of associations with E2Fs during the cell cycle. p130 has been shown to be the major E2F binding partner

present in the G0/G1 phase of the cell cycle, but can also occur throughout the cell cycle (Cobrinik et al., 1993; Hauser et al., 1997; Ikeda et al., 1996; Moberg et al., 1996; Prall et al., 1998; Shiyanov et al., 1996; Smith et al., 1996; Vairo et al., 1995). Complexes between different E2Fs and pRb are mostly found in the G1 to G1/S boundary of the cell cycle (Chittenden et al., 1993; Cobrinik et al., 1993; Schwarz et al., 1993). The interaction timing of E2F with p107 is somewhat different. p107/E2F complexes are predominantly associated with cyclin E/cdk2 during G1 and cyclin A/cdk2 during S phase of the cell cycle (Lees et al., 1992; Mudryj et al., 1991; Shirodkar et al., 1992). Analogous with p107, p130 also binds to these cyclins in complex with E2F on DNA (Cobrinik et al., 1993; Shiyanov et al., 1996). The significance of these different E2F interactions is not fully understood.

1.17 E2F4 and p130 are G0 transcriptional repressors

As noted previously, E2F4 can act as an activator of transcription and induce transformation of cells, but recent data suggests that a more widespread function might be to act with p130 to form a suppresser complex during G0 and early stages during the cell cycle. E2F4 is the predominant E2F during the quiescent state of the cell cycle a time when the genes necessary for the cell to cycle are silent (Leone et al., 1998; Lindeman et al., 1997; Moberg et al., 1996; Smith et al., 1996; Vairo et al., 1995; Verona et al., 1997). Also, in G0 the predominant pocket protein that interacts with E2F is p130 (Cobrinik et al., 1993; Ikeda et al., 1996; Leone et al., 1998; Moberg et al., 1996; Smith et al., 1996; Vairo et al., 1995). The induction of gene expression corresponds to a time when E2F4 no longer binds to its promoter site during G1 progression as revealed by *in vivo* footprinting experiments on the cdk1 and b-Myb enhancer sites (Tommasi and Pfeifer, 1995; Zwicker et al., 1996). This finding is supported by studies on other E2F binding promoter sites that reveal the loss of E2F4 binding at promoter sites during progression of the cell cycle in G1

when gene expression from these sites normally takes place (Johnson, 1995; Johnson et al., 1994; Ohtani et al., 1996; Ohtani et al., 1995; Sears et al., 1997). The disruption of E2F4 binding to DNA and/or the onset of gene expression also equates with the loss of its potential to interact with p130 in a DNA complex (Ikeda et al., 1996; Johnson, 1995; Leone et al., 1998). Furthermore, the availability of both proteins to suppress promoter sites is curtailed dramatically in mid G1 as the protein levels of p130 are reduced and E2F4 is redistributed to the cytoplasmic compartment (Leone et al., 1998; Muller et al., 1997; Verona et al., 1997).

1.18 Cyclin A and E interaction with p107 and p130 can suppress cdk2 activity

The cyclin/cdk2 complex formation with p107 or p130 is facilitated by the spacer region and by an N-terminal motif which is known to bind cyclin A and cyclin E (Adams et al., 1996; Lacy and Whyte, 1997; Smith and Nevins, 1995; Zhu et al., 1995). pRb does not bind stably to cyclin A or E as it lacks a large spacer region. Sites for cyclin A or E interaction with pRb have recently been deduced at a C-terminal region (Adams et al., 1999). As mentioned earlier the act of binding might result in the phosphorylation of the pocket protein resulting in the release of E2F that retains its DNA binding potential (Mayol et al., 1996; Zhu et al., 1995). Another possible outcome of binding is the suppression of cdk2 activity by the pocket protein (Castano et al., 1998; Coats et al., 1999; Woo et al., 1997; Zhu et al., 1995). *In vivo* p130 complex formation with cyclin E/cdk2 during mitogen starvation in p27-/- murine embryonic fibroblasts, is a function normally devoted to p27 (Coats et al., 1999). For p107 and p130, the ability to suppress cdk2 can be mediated by an N-terminal motif that binds to cyclins A and E. (Castano et al., 1998; Woo et al., 1997). The inhibition of kinase activity is a tissue specific and cell cycle dependent phenomenon. p130 and p107 have been shown to direct cdk2 phosphorylation of different

substrates (Hauser et al., 1997). In addition, cyclin E/cdk2 can be found in activated forms with both p107 and p130 during different phases of the cell cycle (Li et al., 1993). For example, a cyclin E/cdk2 activated kinase with p107 and E2F induces transcription from an E2F binding site in the cyclin A gene promoter (Zerfass-Thome et al., 1997). p130 has been shown to be in a complex with E2F and activated cyclin E and cdk2 following cyclin D1 or c-Myc ectopic over expression (Prall et al., 1998). Furthermore, the suppression of cdk2 activity in p27-/- mice occurs only in fibroblastic cells that are in G0 not in hematopoietic cells where cdk2 activity is not blocked in quiescence (Coats et al., 1999).

1.19 Cyclin A/cdk2 regulates the activity of E2F by direct binding

E2F1 and most likely E2F2 and E2F3 are down regulated in a pocket protein independent manner via direct binding to cyclin A. E2F1 contains a cyclin A N-terminal binding motif that is also present on E2F2 and E2F3 (Krek et al., 1994; Xu et al., 1994). When cyclin A binds to E2F1 it recruits its kinase partner cdk2 to target E2F1 and/or its heterodimeric partner Dp-1 for phosphorylation (Kitagawa et al., 1995; Krek et al., 1994; Xu et al., 1994). The act of phosphorylation eliminates the heterodimeric DNA binding activity (Dynlacht et al., 1994; Krek et al., 1994; Xu et al., 1994). This effect occurs during S-phase and is required for cellular division to occur in a correct and timely manner (Krek et al., 1995). This level of E2F regulation is specific to cyclin A targeting of E2F1 as cyclin D or E has no effect (Dynlacht et al., 1994).

To date, a pocket protein independent kinase control of E2F4 has not been published. Lacking the cyclin A binding motif, E2F4 and E2F5 are unaffected by a direct cyclin A/cdk2 kinase activity. E2F4 becomes responsive only when the cyclin A binding domain is transferred to it (Dynlacht et al., 1997). Yet E2F4 can be differentially phosphorylated in a cell cycle dependent manner suggesting phosphorylation by cdks (Vairo et al., 1995). Phosphorylation of E2F4 by cdk2 might be facilitated by p107 and

p130 as kinases associated with these molecules can phosphorylate E2F4 *in vitro* (Ginsberg et al., 1994; Vairo et al., 1995).

1.20 E2F1 can induce apoptosis

Early reports demonstrated that overexpression of E2F1 induces apoptosis in fibroblasts despite its oncogenic properties and its ability to drive cells from quiescence into S-phase (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). E2F1 is the only family member thus far that has been shown to induce apoptosis (DeGregori et al., 1997). Other studies where E2F1 is dysregulated confirm its potential to cause apoptosis. For example, the loss of pRb in mice models ultimately impinges on the control of E2F1 leading to dysregulated growth and apoptosis in different cell types and tissues (Almasan et al., 1995; Macleod et al., 1996; Morgenbesser et al., 1994). This may explain why p53 is also mutated in many pRB mutated tumours.

Gene knockout experiments for E2F1 illustrate that it is an important contributor in potentiating apoptosis and provide a hypothesis for why it occurs (Field et al., 1996; Yamasaki et al., 1996). The thymus of E2F1 knockout mice contains an excess of mature T-cells suggesting a reduced ability of the thymocytes to undergo programmed cell death (Field et al., 1996). Knockout mice are also predisposed to a wide range of tumour formation suggesting an E2F1 role as a tumour suppressor (Yamasaki et al., 1996). This result taken together with the above findings provides a model for E2F1 in the surveillance of proper cellular division as a means to eliminate cells that have lost the ability to cycle normally. Another model to explain the paradox of E2F1's role as a tumour suppressor and oncogene arises from recent experiments with mice generated from crosses between Rb-/+ and E2F1-/- backgrounds. The loss of E2F1 reduces the frequency of pituitary and thyroid tumours that usually occur in Rb-/+ mice; a result that is opposite to what is expected if the previous model is correct (Yamasaki et al., 1998). The simplest

interpretation for these results is a model for E2F1 acting in a tissue specific manner dependent on the amount of pRb that is present in the cell at any given moment.

E2F1 can induce apoptosis in both p53 dependent and independent fashions. The p53 dependent mechanism involves the transactivation potential of E2F1. In this case, it has been ascertained that E2F1 overexpression promotes the synthesis of $p14^{ARF}$ ($p19^{ARF}$ in mice) which is the alternative reading frame gene that is expressed from the $p16^{INK4a}$ gene locus (Bates et al., 1998; Palmero et al., 1998). $p14^{ARF}$ stabilizes the p53 protein by complex formation with Mdm2 and p53 (Pomeratz et al., 1998; Zhang et al., 1998). Mdm2 normally acts in prompting the degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Also, E2F1 may affect the stability of p53 by binding directly to Mdm2 (Martin et al., 1995). E2F1 can induce apoptosis in a p53 independent manner. In this case, E2F1 DNA binding ability, but not its transactivation potential is necessary to promote apoptosis in cells lacking p53 (Hsieh et al., 1997; Phillips et al., 1997).

1.21 The role for E2F in differentiation

Differentiation is a process that down regulates the proliferative ability of cells leading to growth arrest and the expression of genes necessary to alter cell morphology and maintain cells in a state of quiescence. A hint that E2F is involved in differentiation comes from the need to shut off the genes required for cell cycle progression, the potential of E2F to form repressor complexes with pocket proteins at their binding sites and the developmental abnormalities in pocket protein and E2F gene targeting experiments. At G0, E2F4-p130 represents the predominant E2F complex binding to DNA at a time when the bound promoters are turned off (Leone et al., 1998; Lindeman et al., 1997; Moberg et al., 1996; Muller et al., 1997; Smith et al., 1996; Vairo et al., 1995; Verona et al., 1997). Thus, it is expected that the bulk of E2F binding sites would contain these molecules at least during the process of differentiation. This is the case as cells differentiate, E2F

complexes not associated with pocket proteins are lost and complexes containing E2F4 bound to p130 and pRb increase. This transformation has been demonstrated in muscle cell differentiation when rat L_6 myoblasts and mouse C2C12 cells were induced to differentiate (Corbeil et al., 1995; Kiess et al., 1995; Shin et al., 1995). p130-E2F4 complex binding to DNA also increases during neuronal differentiation of the embryonal carcinoma cell line P19 (Corbeil et al., 1995). Melanoma and hematopoietic cell lines obliged to differentiate also exhibit high levels of p130 in complex formation with E2F4 (Ikeda et al., 1996; Jiang et al., 1995; Smith et al., 1996).

Gene targeting experiments for the pocket proteins reveal a preponderance of developmental abnormalities suggesting involvement of the E2F family. In pRb gene knockouts, mice were defective in nervous system and liver hematopoietic cells, erythropoiesis and skeletal muscle development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Zacksenhaus et al., 1996). p107 or p130 targeted mice have no overt phenotypic defects in a 129/Sv:C57BL/6J genetic background, but mice defective in both genes in this background exhibited defective chondrocyte growth and defective bone development (Cobrinik et al., 1996; Lee et al., 1996). In a BALB/cj genetic background p107 -/- mice displayed myeloproliferative disorders and p130-/- animals die in utero with poor spinal cord and dorsal root ganglia development (LeCouter et al., 1998; LeCouter et al., 1998).

E2F5 represents another E2F family member implicated in differentiation. E2F5 is localized in terminally differentiating or differentiated cells during murine embryonic development (Dagnino et al., 1997). In addition, E2F5 is unable to by-pass a p16 block in G1 and induce S-phase in quiescent cells (DeGregori et al., 1997; Lukas et al., 1996; Mann and Jones, 1996). E2F5 targeted gene disruption leads to developmental anomalies. E2F5 null mice exhibit defects in choroid plexus function as evidenced by the excessive

cerebrospinal fluid production suggesting a developmental abnormality in organ physiology control function (Lindeman et al., 1998).

1.22 E2F Promoter binding sites are not equal

Recent data support the hypothesis that E2F function is not redundant despite the ability of the E2F family members to interact with the same binding sites and activate transcription from the same promoters cloned into plasmids. For example, the functional outcome of promoter binding by members of the E2F family may not be identical. As already mentioned E2F4 is the major E2F member present at G0 (Cobrinik et al., 1993; Lindeman et al., 1997; Muller et al., 1997; Smith et al., 1996; Vairo et al., 1995; Verona et al., 1997). At subsequent periods in the cell cycle E2F4 no longer binds to the promoter as evidenced by *in vivo* footprinting experiments on the b-myb and cdc2 promoters (Tommasi and Pfeifer, 1995; Zwicker et al., 1996). On other promoter sites, E2F4 is lost, to be replaced by other E2F family members (Hiyama et al., 1998; Karlseder et al., 1996; Ohtani et al., 1996; Ohtani et al., 1995; Sears et al., 1997; Watanabe et al., 1998). Corroborating this function is the cytoplasmic localization of E2F4 after mid G1 of the cell cycle suggesting that it is no longer active (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). In addition E2F4's role in quiescence is evident as it is the primary E2F that is upregulated and bound to p130 and/or pRb during the induced differentiation of many cell types (Corbeil et al., 1995; Ikeda et al., 1996; Jiang et al., 1995; Kiess et al., 1995; Smith et al., 1996).

There are also differences in the ability of the E2Fs to transactivate genes that are necessary for cell cycle progression. E2Fs are not equal in the potential to induce cellular proliferation. The use of adenoviruses expressing recombinant E2F1 through to E2F5 demonstrated that E2F1, E2F2 and E2F3 share the capacity to induce S-phase in serum starved fibroblasts in contrast to weak induction by E2F4 and E2F5 (DeGregori et al.,

1997). In addition, E2F4 and E2F5 are unable to by pass a p16 block at G1 as opposed to E2F1, E2F2 and E2F3 (Lukas et al., 1996; Mann and Jones, 1996). For E2F5 this function is apparent from results exhibiting the accumulation of E2F5 in differentiating and terminally differentiated tissues in the developing murine embryo (Dagnino et al., 1997). E2F3 has recently been shown to function differently than E2F1 and E2F2 (Leone et al., 1998). In rat fibroblastic cells that cycle from G0 E2F1, E2F2 and E2F3 are expressed and interact with its DNA consensus binding site. However, only E2F3 DNA binding is manifest for cells that continue cycling without re-entering a G0 phase.

Interacting proteins also affect the functional outcome of E2F binding at promoter sites. E2F1, E2F2 and E2F3 interact primarily with pRb and not with p107 or p130, whereas E2F4 and E2F5 interact with all the pocket proteins. This points to differences in terms of interaction with specific E2F sites and the basal transcriptional machinery. Reports indicate that different E2F binding sites on promoters are p107 and p130 specific versus pRb specific. This was revealed from two different experimental approaches. A repetitive immunoprecipitation-PCR procedure (CASTing) to obtain consensus DNA binding sites for different E2F combinations demonstrated that E2F, Dp and pRb proteins influence the selection of E2F binding sites (Tao et al., 1997). In other experiments, primary cells that lack pRb or both p107 and p130, in a 129/Sv:C57BL/6J genetic background, describe the dysregulated gene expression of completely different E2F targets (Hurford et al., 1997). Also, the propensity of different transcription factor binding sites in the vicinity of the E2F sites on different promoters impart a role for co-transactivation or repression. Sp-1 has been described to function in synergy with E2F1, at the thymidine kinase promoter for gene expression to occur whereas E2F4 elicits the opposite effect (Karlseder et al., 1996).

1.23 Statement of purpose

E2F4 is an important E2F family member by virtue of its abundance at any point during the cell cycle and its presence on promoter sites during quiescence. As noted in the introduction, E2F4 is regulated by its interaction with the pocket proteins, pRb, p107 and p130. The p130-E2F4 interaction might have a role in the switch from G0 to G1. The mechanism of regulation is poorly understood. Notably, the contribution of cyclin/cdks to E2F4 regulation in a p130 dependent and independent manner has not been well characterized. The objective of this work is to determine the effect of cdk2 and cdk4 activity on uncomplexed E2F4 or when in complex with p130.

Chapter 2. General Methods

2.1 Cell Culture

2.1.i Cell Culture and Cell Cycle Synchronization

Human cell lines C33A (cervical carcinoma) and ChaGO K1 (bronchogenic carcinoma) were obtained from the American Type Culture Collection (ATTC). Human cell line U7 is a stably transfected cell line overexpressing p130 derived from U2OS (osteosarcoma) (Dr. Susan Lacy). BALB/c-3T3 are murine immortal fibroblast cells (ATTC). SF9 cells are insect cells derived from Spodoptera fragiperda. Mammalian cells were grown in Dulbecco's Eagle media (DMEM) supplemented with 10% fetal bovine serum (Sigma, Gibco), 250U/ml penicillin/streptomycin (Gibco) and 5ug/ml fungizone (Gibco) at 37°C, with 5% CO_2 in a humidified incubator. Adherent cells were detached from dishes by addition of 0.05% trypsin/EDTA (Gibco).

For cell cycle synchronization, BALB/c cells were serum starved in DMEM, with 250U/ml penicillin/streptomycin (Gibco) and 5ug/ml fungizone (Gibco) at 37°C, 5% CO_2 in a humidified incubator. Twenty four hours post starvation the cells were re-stimulated by the addition of 10% fetal bovine serum.

SF9 cells were cultured in Grace's insect cell media (Gibco) supplemented with 10% fetal bovine serum (Sigma, Gibco), 250U/ml penicillin/streptomycin (Gibco), 5ug/ml fungizone (Gibco), 0.07g/L yeastolate and 0.07 g/L lactalbumin at 27°C in an incubator.

2.1.ii Growth of Baculovirus and infection of SF9 cells

Freshly passaged SF9 cells were infected at a multiplicity of infection (M.O.I.) of approximately five to 15 plaque forming units (p.f.u.)/cell for 24 or 48 hours. Viruses were grown in 15 cm dishes by infection of cells for at least 6 days, supernatants containing virus were removed from plates and centrifuged at 2,000 x g in a Sorval table top centrifuge for 10 minutes. Precleared supernatant containing virus was stored at 20°C.

2.1.iii Metabolic labeling

For metabolic labeling of SF9 cells, 3 ml of Grace's methionine minus media and 11μCi ^{35}S-methionine/cysteine (Dupont) was added to 6 cm petri dishes of adherent cells. Cells were allowed to incubate at 27°C for 2 hours. The media was removed and the cells lysed on the dish with 1 ml E1a buffer (50mM Hepes, pH 7.0, 250mM NaCl, 0.1% NP40) (Whyte et al., 1988) for 10 minutes at 4°C. Lysates were cleared by centrifugation in an Eppendorf centrifuge for ten minutes at maximum RPM (12,000 x g). For mammalian cells, 5 ml of methionine/cysteine minus media and 25μCi of ^{35}S-methionine/cysteine (Dupont) was added to 10 cm dishes of adherent cells. Cells were allowed to incubate at 37°C 5% CO_2 in a humidified incubator for 3 hours. The media was removed and the cells lysed on the dish with 1 ml E1a buffer for 10 minutes at 4°C. Lysates were cleared by centrifugation in an Eppendorf centrifuge for ten minutes at maximum RPM.

2.2 Immunoprecipitations and phosphatase treatment

The protein concentrations of clarified radioactive or non-radioactive supernatants of lysed cells were determined by the Bio-Rad protein assay reagent. The supernatants were normalized for protein concentration and immunoprecipitated by addition of the appropriate antibody and protein A Sepharose (Sigma). Rabbit anti-mouse antibody (Ram) was also added when the antibodies for cyclin A (C160), cyclin E (168) and E2F5 (MH-5) were used. Reaction volumes were made up to 1 ml with E1a buffer and incubated with rocking at 4°C for at least 1 hour. Immune complexes collected on protein A Sepharose were washed at least 5 times in E1a buffer.

For phosphatase treatment, 1,200 U of Lambda Protein Phosphatase (New England Biolabs) was added after washing immune complexes, according to the manufacturer's guidelines. The treated immune complexes were incubated at 30°C for 30 minutes and resolved as for untreated complexes.

To resolve immune complexes, protein A Sepharose was resuspended in 60μl of 2X Laemmli sample buffer (0.0625 M Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 1 mmol DTT, 0.0001% bromophenol blue) (Laemmli, 1970) and denatured by heating at 100°C for 3 minutes. The denatured protein samples were loaded on SDS-polyacrylamide gels and separated by electrophoresis.

The p130 antibodies ct/ntp130 are rabbit polyclonals derived from epitopes at the C-terminal and N-terminal regions of p130 (Lacy and Whyte, 1997). Z83 is a monoclonal p130 antibody (Li et al., 1993). 168 and C160 are cyclin E and cyclin A monoclonals (Giordano et al., 1989; Lacy and Whyte, 1997). The polyclonals Dp-1 (K20), cyclin D3 (C-16), E2F4 (C20), p130 (C20) and monoclonal E2F5 (MH-5) are from Santa Cruz Biotechnology. The cyclin D1 monoclonal DCS-G was a kind gift from Dr. Jiri Bartek (Copenhagen, Denmark) and the cyclin D1 polyclonal employed for Western blots was

from Upstate Biotechnology. The anti-Ha monoclonal antibody, 12CA5 was generously provided by Dr. John Hassell (McMaster University).

2.3 *In vitro* kinase assays

After the final wash with E1a buffer, the protein A Sepharose was resuspended in 50µl of kinase buffer (50mM Tris pH 7.4, 10mM $MgCl_2$, 5mM $MnCl_2$) with 5µCi γ32P-ATP and incubated at room temperature for at least 15 minutes. The reaction was stopped with the addition of 2X Laemmli sample buffer. For re-immunoprecipitation assays of ^{32}P labeled proteins 100µl of SDS release buffer (50mM Hepes pH 7.0, 250 mM NaCl, 1% SDS, 0.5mM DTT) was added at the end of the kinase incubation and the mixture was heated at 100°C for 10 minutes. The samples were then cleared by centrifugation for ten minutes in an Eppendorf centrifuge. The supernatants, consisting of the proteins from the denatured immune complex, were diluted in a final volume of 1.4 ml with E1a buffer and employed in another round of immunoprecipitation, overnight.

2.4 Fluorography and autoradiography

To process SDS-polyacrylamide gels for fluorography, gels were sequentially immersed in destain (7% acetic acid, 15% methanol) for 1 hour, followed by 15 minutes in DMSO and 45 minutes in PPO (2,5-diphenyloxazole)/DMSO (10% PPO w/v). This process was followed by a 30 minute rinse in water. Gels were placed on Whatman paper and dried prior to exposure to film. For autoradiography of gels containing ^{32}P, gels were immersed in water for ten minutes, placed on Whatman paper and dried. In both cases, the gels were exposed at -80°C to Kodak X-omat film.

2.5 Western blot Analysis

Following the separation of proteins by gel electrophoresis, gels were wet-blotted to nitrocellulose (Amersham) in transfer buffer (380 mM glycine, 50 mM Tris, 10% methanol) at 20 volts overnight or 36 volts for at least 3 hours. Blots were blocked by incubating in 10% w/v nonfat powdered milk in Tris Buffered Saline-Triton (TBST) (136 mM NaCl, 20 mM Tris pH 7.4, 0.15% Triton X-100) and 0.02% sodium azide for at least 4 hours. Post blocking, primary antibody was added to the blocking mixture and incubated overnight. Blots were washed three times in TBST by rocking for ten minutes. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted 1/5000 (Signal Transduction) for 1 hour in 10% w/v nonfat milk in TBST. Blots were washed three times as above and twice for ten minutes each time in PBS. At the end of the wash cycle, after excess liquid was removed the blots were developed by addition of ECL substrate (Amersham) and exposed to film.

2.6 Purification of plasmid DNA

2.6.i Small scale

An isolated bacterial colony containing the plasmid DNA of interest was used to inoculate 3.0 ml of Luria-Bertani medium (LB) (1.0% w/v bacto-tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl) containing 0.1mg/ml ampicillin. Cells were grown overnight. The DNA was isolated according to the Birnboim and Doly method with minor modifications (Birnboim and Doly, 1979). 1.5 ml of overnight culture was added to a microcentrifuge tube, pelleted and resuspended repeated in 100 µl of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0). 200 µl of Solution II was added to the suspension (0.2 M NaOH, 1% SDS) and the mixture was incubated on ice for 5 minutes. 150 µl of Solution III (3.0 M potassium acetate pH 4.8) was added and the mixture was

incubated for a further five minutes. The resulting precipitate was pelleted by centrifugation for five minutes and the supernatant decanted to a fresh micro centrifuge tube and extracted with an equal volume of equilibrated phenol. The aqueous phase was transferred to a fresh microcentrifuge tube and nucleic acids were precipitated by adding 1 ml of 95% ethanol. After standing for 5 minutes, the tube was centrifuged for 10 minutes to pellet the precipitated DNA. The ethanol was decanted, the tube inverted for proper drying and the contents resuspended in 40 µl of water.

2.6.ii Large scale

Plasmids were propagated in DH5α (E. Coli strain) in 500 ml 2.5X LB with 0.1mg/ml ampicillin overnight. The cultures were centrifuged at 7000 RPM in a Beckman J2-21 centrifuge for 10 minutes at 4°C and the cell pellet lysed by the method of Birnboim and Doly using the small scale purification procedure with modifications. The cell pellet was resuspended in 12 ml of Solution I, 24 ml of Solution II and 15 ml of Solution III. After incubation with Solution III the suspension was centrifuged at 7000 RPM for ten minutes. The supernatant was decanted and precipitated with 0.6 volume of isopropanol for 5 minutes and centrifuged at 7000 RPM for 15 minutes. Pellets were resuspended in water with CsCl added to a final concentration of 1mg/ml and 400 µl of 25mg/ml of ethidium bromide. The suspension was centrifuged for 3 minutes at 2,000 g in a Sorval table top centrifuge and the supernatant loaded into Beckman ultracentrifuge tubes. The tubes were centrifuged for 17 hours in a Beckman ultracentrifuge at 55,000 RPM in a Vti65.1 rotor.

After centrifugation, the plasmid DNA band was removed by syringe and needle and the ethidium bromide was removed from the DNA by butanol extraction. The DNA was precipitated by addition of 1/10 volume of 3M sodium acetate and 5 volumes of 95% cold ethanol. DNA was collected by centrifugation at 2,000 g for 20 minutes and

resuspended in 70% cold ethanol. The DNA was collected once more by centrifugation for 10 minutes and resuspended in 500 µl of water. DNA was quantitated by a spectrophotometer reading to give O.D. 260.

2.7 Transient transfections

Transient transfections of mammalian cells employed the calcium phosphate precipitation technique with certain modifications (Graham and Van Der Eb, 1973). 10 µg of plasmid DNA (unless stated otherwise) was mixed with 100 µl of 2.5 M $CaCl_2$ and added to 900 µl of water dropwise. This mixture was slowly bubbled into 1ml of HEBS (42 mM Hepes pH 7.15, 273 mM NaCl, 0.28 mM Na_2HPO_4, 10 mM KCl, 10 mM Dextrose). Precipitates were allowed to sit for 38 minutes before the addition of 1 ml to one 10 cm plate of mammalian cells. The cells used for transfections were passaged the day before and had the media replaced at least 1 hour before the DNA-calcium phosphate precipitates were added. Fifteen hours post transfection, the cells were rinsed twice with PBS and incubated with fresh medium for at least 24 to 30 hours before harvesting.

2.8 Polymerase chain reaction (PCR)

Oligonucleotide primers were synthesized and purified by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University (MOBIX). The PCR conditions typically used a 2 minute denaturation step at 94°C, a 1 minute annealing step at 50°C and an extension step at 72°C for 3 minutes repeated for 30 cycles. The PCR products were analyzed on agarose gels stained with ethidium bromide and illuminated under ultraviolet light.

2.9 Plasmids

The plasmids pBSK DP-1, pCMVHADP-1, pCMVHAE2F-1, pCMVHAE2F-2, pCMVHAE2F-3, pCMVHAE2F-4, pCMVHAE2F-5 and pBSKHAE2F-4 were provided by Dr. Kristian Helin (Milan, Italy). The E2F reporter plasmid $E2F_4$Cat was provided by Dr. Ali Fattaey (ONYX). The cyclin expression constructs Rc/cycA, Rc/cycE, Rc/cycD and Rc/cycD3 were provided by Dr. Robert Weinberg (Whitehead Institute) and pcmvCycE was provided by Dr. James Roberts (Seattle, Washington). The cdk plasmid constructs pCMVcdk2wt, pCMVcdk2dn, pCMVcdk4wt and pCMVcdk4dn were provided by Dr. Ed Harlow (Massachusetts General Hospital). The p130 expression constructs CMVp130, 372C, 542C, 602C, 820C and 372C-BN were created by Greg Culp. p130 (Δ620-697), designated as (-sp)p130, was made by Dr. Susan Lacy. The cloning expression vector pcDNA3 was from Invitrogen and the luciferase control vector pGL3 Control was from Promega. The baculovirus transfer vector pVL1393 and pVL1392 and linearized baculovirus DNA (BaculoGold) were from Pharmingen.

2.10 DNA restriction and modifying enzymes

Restriction endonucleases, T4 DNA ligase and E. Coli Klenow fragment were purchased from Pharmacia, New England Biolabs or Boehringer Mannheim and used according to the manufacturers guidelines.

2.11 Construction of recombinant plasmids

2.11.i Construction of E2F4 Ha-tagged expression vectors

The E2F Ha-tagged expression plasmids were produced from pBSK HaE2F-4 by PCR amplification. cDNAs encoding C-terminal truncated proteins made use of a common 5' primer: T7 5'-TAATACGACTCACTATAGG-3' and different 3' primers each containing a stop codon and Xba I restriction endonuclease site:

mutant 382CT: 5'-GCTCTAGACTAACGAAGCAGAGGGGCA-3'

mutant 305CT: 5'-GCTCTAGACTACAGCAGGGCAGAAGAC-3'

mutant 202CT: 5'-GCTCTAGACTATGAGCTCCATGCCTCCTT-3'

mutant 176CT: 5'-GCTCTAGACTACTGCCCATTGAGACCCTC-3'

The amplified PCR products were digested by HindIII and XbaI and ligated into the HindIII/XbaI digested pcDNA3 expression vector. The HindIII digest retains the N-terminal Ha epitope and the E2F4 amino acid sequences in a correct reading frame.

The cDNAs encoding N-terminal truncated proteins made use of a common 3' primer: T3 5'-ATTAACCCTCACTAAAG-3' and different 5' primers each containing a BamHI restriction endonuclease site:

mutant 44NT: 5'-CGCGGATCCCTGGCAGCTGACACCCTAGCT-3'

mutant 84NT: 5'-CGCGGATCCGGGCCTGGCTGCAATACCCGG-3'

mutant 128NT: 5'-CGCGGATCCACAGAGGACGTGCAGAACAGC-3'

mutant 179NT: 5'-CGCGGATCCCAGATTCACCTGAAGAGT-3'

The amplified PCR products were digested by BamHI and Xba I and ligated into the BamHI/XbaI digested pCMVHAE2F-4 vector. The BamHI digest of the vector retains sequences encoding N-terminal Ha epitope in frame with the newly added PCR product.

E2F4 plasmid constructs with both C and N-terminal deletions made use of a common 3' primer: 5'-GCTCTAGACTAACGAAGCAGAGGGGCA-3' and different 5' primers:

mutant 128NT\382CT: 5'-CGCGGATCCACAGAGGACGTGCAGAACAGC-3'

mutant 179NT\382CT: 5'-CGCGGATCCCAGATTCACCTGAAGAGT-3'

The PCR products were digested with BamHI and XbaI and ligated into the BamHI/XbaI digested pCMVHAE2F-4 vector.

2.11.ii p130 C-terminal mutants

The cDNAs encoding C-terminal p130 truncated proteins were produced by PCR amplification of CMVp130 making use of a common 5' primer containing a BamHI site 5'CGGGGTACCGGATCCGCTATGGCGTCGGGAGGTGAC-3' and different 3' primers containing a stop codon and Not1 restriction endonuclease site:

mutant 932N: 5'-GCTCTAGACTATATCAAAACACTTCTATA-3'

mutant 835N: 5'-GCTCTAGACTACCTCAATTCATCTGAA-3'

The resulting PCR products were digested by BamHI and NotI and ligated into pcDNA3 that had been digested by BamHI/Not1.

2.11.iii Dp-1 expression vector

The pcmvDp-1 plasmid was produced by digesting pBSKDP-1 by EcoRI and ligating into the EcoRI digested pcDNA3 vector.

2.11.iv Cloning of recombinant baculovirus transfer vectors

pVLE2F5 was cloned by digestion of pCMVHAE2F- 5 with BamHI and ligation into BamHI digested pVL1393. pVLDp-1 was cloned by digestion of pBSKDP-1 with EcoRI and ligation into pVL1393. pVLE2F4-Ha was cloned by digestion of pBSKHAE2F-4 with EcoRI and ligation into EcoRI digested pVL1392.

2.12 Generation of recombinant baculoviruses

Recombinant baculoviruses for E2F4-Ha, Dp-1 and E2F5 were generated by the co-transfection method. 0.5μg of linearized baculovirus DNA (BaculoGold, Pharmingen) containing a lethal deletion and unable to code for viable virus was mixed with 5μg of recombinant baculovirus transfer vector (pVLE2F5, pVLDp-1 or pVLE2F4-Ha). The mixture was allowed to stand for 5 minutes before the addition of 1 ml of transfection buffer (25 mM Hepes pH 7.1, 125 mM $CaCl_2$, 140 mM NaCl). The media from a 6 cm dish of 80% confluent SF9 cells was replaced with 1 ml of Grace's supplemented media. The DNA/buffer mixture was added to the dish dropwise with swirling and incubated at 27°C for four hours. Co-transfection with the complementing plasmid, the transfer vector plasmid, rescued the lethal deletion of the viral DNA (BaculoGold, Pharmingen) resulting in the production of viable virus particles expressing the gene of interest in the transfected SF9 cells. After incubation with the transfection solution the media was removed, the cells rinsed and 3 ml of Grace's supplemented media was added. After 5 days the virus containing media was harvested by pelleting dead cells by centrifugation.

2.13 Recombinant baculoviruses

Cyclin D1, D2 and D3 encoding viruses were provided by Dr. Charles Sherr (Memphis, Tennessee). Cyclin A and cyclin E and cdk2 were provided by Dr. David Morgan (San Francisco, California). The baculovirus encoding p130 and 372C (containing p130 amino acids 372 to 1139) were constructed by Dr.Yun Li.

2.14 Electrophoretic Mobility Shift Assays (EMSA)

2.14.i Annealing of complementary oligonucleotides

To obtain double stranded wildtype or mutant E2F oligonucleotides 1.75 μg of each single stranded complementary oligonucleotide was added to annealing buffer (400 mM Tris pH 8.0 and 100 mM $MgCl_2$) to a final concentration of 5 μl and the mixture heated to 100°C for five minutes. The annealing reaction mix was cooled gradually overnight to room temperature. The double stranded wildtype E2F oligonucleotide was made from the complementary base sequences 5'-ATTTAAGTTTCGCGCCCTTTCCAA-3' and

5'-TTGGAAAGGGCGCGAAACTTAAAT-3'. The mutant double stranded E2F oligonucleotide was made from the complementary base sequences 5'-ATTAAGTTTCGATCCCTTTCTCAA-3' and 5'-TTGAGAAAGGGATCGAAACTTAAT-3'.

2.14.ii ^{32}P-labeling of double stranded oligonucleotides

To 100 ng of double stranded oligonucleotide 10X T4 polynucleotide kinase buffer (NEB), 100 μCi of γ^{32}P-ATP and 20 units of T4 polynucleotide kinase (NEB) was added to a final reaction volume of 20 μl. The labeling reaction was incubated at 40 minutes at 37°C. After incubation tracking dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 1% Ficoll 400, 10 mM EDTA pH 8.0) was added and the labeled oligonucleotide was electrophoresed on a 8% polyacrylamide gel buffered with 0.5 TBE at 180 volts for 2 hours. At the end of the run the predominantly labeled oligonucleotide band was cut from the gel, minced, resuspended in 0.5 M ammonium acetate and rocked overnight at room temperature. The labeled double stranded oligonucleotide was extracted with

phenol/chloroform extracted, precipitated with ethanol and the pellet resuspended in 40µl of H_2O.

2.14.iii Isolation of nuclear and cytoplasmic Fractions

Subcellular fractionation of cells was performed using hypotonic lysis (Verona et al., 1997). Cells in 10 cm dishes were washed once with PBS. After washing, 1 ml of PBS was added and the cells scraped with a rubber policeman and pelleted at 1000 RPM for ten minutes in a Sorval table top centrifuge. The pellet was resuspended in two packed cell volumes of hypotonic buffer (10 mM Hepes pH7.5, 10 mM KCl, 3mM $MgCl_2$, 1mM EDTA pH 8.0, 10 mM NaF, 0.1mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT)) and incubated on ice for five minutes. The nuclei were pelleted from the suspension by centrifugation at 1000 RPM. The supernatant containing the cytoplasmic fraction was decanted into a fresh tube. The nuclei were washed twice in hypotonic buffer containing 0.05% Nonidet P-40 (BDH). After washing, the nuclei were lysed in three packed cell volumes of lysis buffer (0.5 M KCl, 35% glycerol, 100 mM Hepes pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 5 mM NaF, 1 mM PMSF, 1 mM DTT) for ten minutes on ice. The nuclei were clarified by centrifugation in a Beckman ultracentrifuge at 25,000 RPM in a SWTi55 Beckman rotor for twenty minutes. The cytoplasmic fraction was supplemented with glycerol to 35%, rocked gently for ten minutes to ensure proper mixing and clarified by spinning in an Eppendorf centrifuge at top speed for 30 minutes at 4°C. The protein concentrations were determined by the Bio-Rad protein assay reagent.

2.14.iv Binding reaction and electrophoresis

To a total volume of 19 µl, in an Eppendorf tube the following were added: 2 to 10µg of nuclear or cytoplasmic extract, 2µg of poly (dI-dC) at 1µg/µl (Boeringher Mannheim), 4 ml of 5X binding buffer (250 mM KCl, 100 mM Hepes pH 7.4, 5 mM $MgCl_2$, 5 mM EDTA pH 8.0, 42.5% glycerol) and 1µl of mutant unlabeled double stranded E2F oligonucleotide at 75 ng/µl. To test for the presence of specific proteins 1µl of appropriate antibody was added. To test for specificity of binding 1 µl of unlabeled wildtype double stranded E2F oligonucleotide at 75 ng/µl was added. The binding reaction mixture was incubated at 4°C for ten minutes. After incubation 1 µl of ^{32}P-end labeled double stranded wildtype E2F oligonucleotide was added (representing an activity of at least 10,000 cpm) and incubated for a further 10 minutes. The samples were then electrophoresed at 180 V in 5% polyacrylamide gels buffered with 0.25 X TBE (22 mM Tris, 22 mM borate, 0.5 mM EDTA). The gel was washed for ten minutes in water, blotted on Whatman paper, dried and exposed to film.

2.15 Chloramphenical acetyltransferase (CAT) assays

C33A cells grown in 10 cm dishes were transfected as described above for transactivation assays. Cells were transfected with 5µg of reporter plasmid $E2F_4$Cat containing four tandem E2F-binding sites from the E2A promoter (-71 to -54) (Loeken and Brady, 1989) to measure the E2F4 dependent transactivation. 2 µg of a luciferase expression plasmid pGL3-Control was transfected as an internal control to normalize for transfection efficiency. pcDNA3 or pCMV was added to adjust for total amount of transfected plasmid and 5 to 10 µg of the expression plasmids under study were added in any one experiment.

The transfected cells were washed twice with PBS and lysed with 100 µl of luciferase extraction buffer (100 mM potassium phosphate pH 7.8, 1mM DTT) with three cycles of freeze-thawing in liquid nitrogen and 37°C water bath. The cellular debris was pelleted and the supernatant assayed for CAT (chloramphenical acetyltransferase) and luciferase activities.

The CAT reaction was performed in a total volume of 180 µl with 70 µl of sample, 10 µl of 10 mM Acetyl CoA (Pharmacia), 4 µl of dichloroacetyl-1,2^{14}C-chloramphenicol (New England Nuclear) and 96 µl of 250 mM Tris pH 7.5 at 37°C for 90 minutes. The reaction was stopped by adding 1 ml of ethyl acetate. After stopping the reaction, the tube was centrifuged for 5 minutes and 900 µl of the upper layer containing the acetylated chloramphenicol was added to a fresh tube. The sample was allowed to evaporate and the residue resuspended in 20 µl of ethyl acetate and spotted on Whatman TLC plates in 5µl aliquots. The plate was developed in a 19:1 chloroform:methanol chamber with a total volume of 200 ml. The plates were exposed to a phosphoimager screen and the relative intensities noted.

For transfection efficiency the luciferase activity was determined employing 20 µl of sample in 360 µl of Assay Buffer containing 0.5 mM ATP (Pharmacia), 0.25 mM Coenzyme A (Sigma), 33 mM DTT and 1 times 2.5X Assay buffer (50 mM Tricine, 2.675 mM glycylglycine, 6.675 mM $MgSO_4$, 0.25 mM EDTA). 100 µl of 62.5 µM luciferin (Sigma) in 2.5X assay buffer was injected into the sample in Assay Buffer and the activity was measured by a Berthold luminometer. After normalization of the CAT values the results for at least three different experiments were averaged and presented as a relative activity. The relative activity is expressed with standard error of the mean using the formula $[n\Sigma x^2-(\Sigma x^2)/n^2]^{1/2}$, where n is the number of different experiments and x is the value for the relative activity.

2.16 Phosphopeptide Mapping

Phosphopeptide mapping was performed by Dr. Peter F.M. Whyte (Lacy and Whyte, 1997).

Chapter 3. Cyclin D/cdk4 regulation of E2F4 DNA binding potential

3.1 Introduction

The serendipitous finding that cyclin D1 interacts directly with E2F4 initiated this study. The intention was to test the hypothesis that members of the cyclin D family of proteins might control the activity of the E2F family of transcription factors by direct binding. In particular the regulation of E2F4 function by cyclin D1 targeted phosphorylation was examined. Cyclin A is known to regulate E2F1 and possibly E2F2 and E2F3 in a cdk2 dependent manner (Krek et al., 1994; Krek et al., 1995; Xu et al., 1994). However, cyclin A and cyclin E do not interact directly with E2F4. For the D-type cyclins, reports indicate they can modulate the activity of E2Fs indirectly by binding to and targeting pocket proteins for phosphorylation with their kinase partner (Beijersbergen et al., 1995; Dowdy et al., 1993; Kato et al., 1993; Matsushime et al., 1992; Xiao et al., 1996). Cyclin D1 can also regulate the activity of other transcription factors in a cdk independent manner (Ganter et al., 1998; Inoue and Sherr, 1998; Neuman et al., 1997; Zwijsen et al., 1998; Zwijsen et al., 1997).

The E2F4 region that interacts with cyclin D3 also was mapped. Cyclin D3 is highly homologous to the other D-type cyclins sharing an average of 57% identity with the entire coding region and 78% within the cyclin box domain (Xiong et al., 1992). Cyclin D3 also exhibits a certain amount of functional redundancy. Like cyclin D1 and D2, cyclin D3 can accelerate the progression through G1 from quiescence or in actively proliferating fibroblast cells (Ando et al., 1993; Herzinger and Reed, 1998; Resnitzky et al., 1994) and is essential for cell cycle progression as the microinjection of neutralizing cyclin D3 antibodies results in the inhibition of the G1/S transition in primary cell lines (Baldin et al., 1993; Bartkova et al., 1998; Lukas et al., 1995; Lukas et al., 1994; Quelle et al., 1993).

On the other hand cyclin D3 may have different properties from cyclin D1. Cyclin D3 expression, unlike cyclin D2 and D1, is elevated in many quiescent differentiated cell types, in several tissues and in cell lines induced to differentiate (Bartkova et al., 1998; Kiess et al., 1995; Rao and Kohtz, 1995). Mapping the cyclin D1 and D3 binding sites on E2F4 might lead to insights on their differential properties.

3.2 Results

3.2.i The cyclin D family interacts *in vitro* with the E2F family of transcription factors

The potential of cyclin D1 and cyclin D3 to bind directly to the E2F proteins, without bridging molecules, was tested by relying on a system employing baculovirus expressing recombinant proteins. For this purpose, recombinant baculovirus expressing the E2F4-Ha, E2F5 and Dp-1 proteins were made. The expressed recombinant proteins were observed in the infected lysate when immunoprecipitated by an appropriate antibody (Fig. 3.1A, lanes 1,2,4,6). The E2F4 expressed protein contains an Ha (hemagglutinin) epitope facilitating its immunoprecipitation by the Ha monoclonal antibody 12CA5 (Fig. 3.1A, lane 1). No proteins were immunoprecipitated from the uninfected lysates suggesting the antibodies do not cross react with endogenous SF9 proteins (Fig. 3.1A lanes 8,9,10,11,12). The recombinant viruses in Figure 3.1 were utilized in co-infections with baculovirus expressing cyclin D1 in binding studies. The binding studies made use of SF9 cells infected with E2F4-Ha, E2F5 or Dp-1 in the presence or absence of cyclin D1 expressing virus. Proteins from these lysates were immunoprecipitated with the indicated antibodies and probed with a polyclonal cyclin D1 antibody (Fig. 3.1B). Cyclin D1 interacted with only coinfected E2F4-Ha, E2F5 and Dp-1 proteins (Fig. 3.1B, lanes 6,9,12 compared with lanes 19,21,23).

Figure 3.1 Cyclin D1, D2 and D3 associate *in vitro* with E2F4. (A) *In vitro* protein expression of E2F4-Ha, E2F5 and Dp-1. ^{35}S met/cys- labeled SF9 cell lysates uninfected (lanes 8-12) or infected with baculovirus expressing E2F4-Ha (lanes 1-3), E2F5 (lanes 4 and 5) and Dp-1 (lanes 6 and 7) were immunoprecipitated with the indicated antibodies, separated by electrophoresis on an SDS-10% polyacrylamide gel and visualized by fluorography. (B) *In vitro* association of cyclin D1 with E2F4-Ha, E2F5 and Dp-1. SF9 cells were uninfected (lanes 24-27) or infected with baculovirus expressing cyclin D1 (lanes 1-4 and 14-17), E2F4-Ha (lanes 18 and 19), E2F5 (lanes 20 and 21) and Dp-1 (lanes 22 and 23) or cyclin D1 with E2F4-Ha (lanes 5-7), E2F5 (lanes 8-10) and Dp-1 (lanes 11-13). Immunoprecipitated as indicated, separated by electrophoresis on SDS-10% polyacrylamide gels, blotted and probed for cyclin D1 (Upstate Biotechnology). (C) Cyclin D2 and D3 associate *in vitro* with E2F4-Ha. SF9 cells were uninfected (lanes 10-12); or infected by baculovirus expressing cyclin D2 and cyclin D3 (lanes 1-3), and E2F4-Ha (lanes 4-6) or by cyclin D2 and cyclin D3 with E2F4-Ha (lanes 7-9) immunoprecipitated as indicated, separated by electrophoresis on SDS-10% polyacrylamide gels, blotted and probed for cyclin D2 and cyclin D3 respectively (DSC-5 and DSC-22). (D) E2F4 associates *in vitro* with cyclin D1, D2 and D3. SF9 cells were uninfected (lanes 17-20); or infected by baculovirus expressing cyclin D1 (lanes 1 and 2), cyclin D2 (5 and 6), cyclin D3 (lanes 9 and 10) and E2F4-Ha (lanes 13-16); or by E2F4-Ha with cyclin D1 (lanes 3 and 4), cyclin D2 (lanes 7 and 8) and cyclin D3 (lanes 11 and 12) immunoprecipitated by the indicated antibodies, separated by electrophoresis on SDS-7.5% polyacrylamide gels, blotted and probed for E2F4 (Santa Cruz).

The feasibility of cyclin D2 and D3 interacting with E2F4 was also assessed. SF9 cells were infected with recombinant virus expressing cyclin D2 or D3 in the presence or absence of E2F4-Ha expressing virus. Proteins from infected lysates were immunoprecipitated in parallel for cyclin D2, cyclin D3 and E2F4. The immunoprecipitations were Western blotted for cyclin D2 and D3. Cyclin D2 and D3 are shown to interact with E2F4 (Fig. 3.1C, lanes 7-9 versus lanes 4-6). The reciprocal experiments for the interaction of D-type cyclins with E2F4 were performed to confirm the previous results. SF9 cells were infected with baculovirus expressing cyclins D1, D2 or D3 in the presence or absence of baculovirus expressing E2F4. Proteins from infected lysates were immunoprecipitated for E2F4 with anti-Ha, cyclin D1, D2 and D3 in parallel. The ability of E2F4 to bind the cyclin D family was tested by probing a Western blot for E2F4 (Fig. 3.1D). As before E2F4, co-immunoprecipitated with the D-type cyclins only when the proteins were co-expressed (Fig. 3.1D, lanes 3,4,7,8,11,12). These results suggest that the D-type cyclins can interact with E2F4 and that cyclin D1 can interact with E2F5 and Dp-1.

The potential of cyclin D3 to bind to E2F5 and Dp-1 was tested, as was done for cyclin D1, using baculovirus expressing recombinant protein. SF9 cells were infected with baculovirus expressing recombinant cyclin D3, Dp-1 or E2F5, or cyclin D3 together with Dp-1 or E2F5. Cellular lysates of infected cells were immunoprecipitated for cyclin D3 and E2F5 or Dp-1 and Western blotted for cyclin D3 (Fig. 3.2A,B). Cyclin D3 interacted with E2F5 or Dp-1 when they were co-infected (Fig. 3.2A,B compare lane 5 with lane 8).

3.2.ii Cyclin D1 interacts *in vivo* with the E2F family of transcription factors

The *in vivo* potential of cyclin D1 to interact with the E2F transcription family was next tested. Cyclin D1 is expressed early in G1 shortly after mitogen stimulation. It is a

Figure 3.2 Cyclin D3 associates *in vitro* with E2F5 and Dp-1. (A) and (B) SF9 cells were uninfected (lane 10,11,12) or infected with baculovirus expressing cyclin D3 (lane 1,2,3) and E2F5 or Dp-1 (lane 4,5,6) and cyclin D3 co-infected with E2F5 or Dp-1 (lane 7,8,9). Cellular lysate was immunoprecipitated, for the indicated proteins, by rabbit anti-mouse (Ram), anti-E2F5 (MH-5), anti-Dp-1 (K20) and anti-cyclin D3 (C-16). Immunoprecipitates were separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for cyclin D3 (C-16).

regulatory subunit for cdk4 and cdk6 and is thought to target pRb for phosphorylation and inactivation (Sherr, 1996). The *in vivo* system made use of CMV promoter based plasmids expressing cyclin D1 and Ha epitope tagged E2F family members. To test the *in vivo* potential of cyclin D1 to interact with E2F1 through to E2F4, C33A cells were transiently transfected with plasmids expressing E2F1-Ha, E2F2-Ha, E2F3-Ha and E2F4-Ha with and without cyclin D1. The immunoprecipitations were probed for cyclin D1 (DSC-6). Cyclin D1 co-immunoprecipitated the E2Fs immunoprecipitated by 12CA5 anti-Ha in co-expressing cell lysates (Fig. 3.3A lanes 4,6,8,10). The endogenous cyclin D1 in C33A cells was not visible and did not contribute to binding with the exogenous E2Fs (Fig. 3.3A, lane 19). The interaction of cyclin D1 with E2F1, E2F2, and E2F3 was not as efficient as with E2F4 (Fig. 3.3A, lanes 4,6,8 compared with lane 10). In each immunoprecipitation, the cell lysate was normalized for protein content and the expression of E2F1, E2F2 and E2F3 was comparable to the E2F4 level in the lysate (data not shown). The potential of cyclin D1 to bind Dp-1 and E2F5 was also assessed employing transient transfections as described above (Fig. 3.3B). As with the previous results, E2F5 and Dp-1 also bind to cyclin D1 (Fig. 3.3B, lanes 8,10). In addition, the results also indicate that E2F5 and Dp-1 are more efficient in binding to cyclin D1 than E2F1, E2F2 and E2F3 and as efficient as E2F4 (Fig. 3.3A, lanes 4,6,8 compared with Fig. 3.3B, lanes 8,10). Taken together these results suggest that E2F1, E2F2 and E2F3 may interact indirectly with cyclin D1 as compared to E2F4, E2F5 and Dp-1.

3.2.iii Cyclin D3 interacts *in vivo* with the E2F family of transcription factors

The *in vivo* potential of cyclin D3 to interact with the E2F protein family was assessed by employing CMV based plasmids expressing E2F1 through to E2F5 and Dp-1. C33A cells were transiently transfected by plasmids expressing E2F1-Ha, E2F2-Ha, and

Figure 3.3 Cyclin D1 associates with the E2F family of transcription factors *in vivo*. (A) Cyclin D1 associates *in vivo* with E2F1, E2F2, E2F3 and E2F4. C33A cells were untransfected (lanes 19 and 20) or transfected with 5ug each of Rc/cycD (lanes 1,2,11 and 12), pCMVHAE2F-1 (lanes17 and18), pCMVHAE2F-2 (lanes15 and16), pCMVHAE2F-3 (lanes 13 and 14) or by Rc/cycD co-transfected with pCMVHAE2F-1 (lanes 3 and 4), pCMVHAE2F-2 (lanes 5 and 6), pCMVE2F-3 (lanes 7 and 8) or pCMVHAE2F-4 (lanes 9 and 10). Proteins from cell lysates were immunoprecipitated as indicated, separated by electrophoresis on an SDS-10% polyacrylamide gel, blotted and probed for cyclin D1 (DSC-6). (B) Cyclin D1 associates *in vivo* with E2F5 and Dp-1. C33A cells were transiently transfected by Rc/cycD (lanes 1 and 2), pCMVHAE2F-5 (lanes 3 and 4) and pCMVHADp-1 (lanes 5 and 6) or Rc/cycD together with pCMVHAE2F-5 (lanes 7 and 8) and pCMVHADp-1 (lanes 9 and 10). Proteins from cell lysates were immunoprecipitated as indicated, separated by electrophoresis on an SDS-10% polyacrylamide gel, blotted and probed for cyclin D1 (Upstate Biotechnology). IgH and IgL denotes nonspecific immunoglobulin heavy and light chain binding.

Figure 3.4 Cyclin D3 interacts with E2F4, E2F5 and Dp-1 *in vivo* (A) C33A cells were transiently transfected with plasmids expressing cyclin D3 (lanes 1 and 2), E2F1-Ha (lanes 3 and 4), E2F2-Ha (lanes 5 and 6) and E2F3-Ha (lanes 7 and 8) and cyclin D3 together with E2F1-Ha (lanes 9 and 10), E2F2-Ha (lanes 11 and 12) or E2F3-Ha (lanes 13 and 14). Proteins from transfected lysate were immunoprecipitated in parallel for cyclin D3 (C-16) and Ha-tagged E2Fs (12CA5), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for cyclin D3 (C-16). (B) C33A cells were transiently transfected with E2F4-Ha (lanes 1 and 2), E2F5-Ha (lanes 3 and 4), and Dp-1-Ha (lanes 5 and 6), and cyclin D3 together with E2F4-Ha (lanes 7 and 8), E2F5-Ha (lanes 9 and 10), or Dp-1-Ha (lanes 11 and 12). Proteins from transfected lysate were immunoprecipitated for Ha-tagged proteins (12CA5) and cyclin D3 (C-16), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for cyclin D3 (C-16).

E2F3-Ha with and without cyclin D3. Cyclin D3 and E2F were immunoprecipitated from transfected cellular lysates. The immunoprecipitations were analyzed for the presence of cyclin D3 by Western blotting. In contrast to cyclin D1 (Fig. 3.3A), cyclin D3 did not interact with E2F1, 2 and 3 (Fig. 3.4A lanes 10,12,14). The same method was employed to illustrate the cyclin D3 interaction with Dp-1, E2F4 and E2F5 *in vivo* (Fig 3.4B lanes 10,12,14). The association of cyclin D3 with E2F4 and Dp-1 did not appear to be as efficient as with E2F5 (Fig. 3.4A, lanes 8 and 12 compared with lane10). The weak binding with Dp-1 suggests an indirect interaction, perhaps facilitated by E2F4 or E2F5. Thus, a possible explanation for E2F1, E2F2 and E2F3 negative interactions with cyclin D3 is the requirement for a direct Dp-1 interaction.

3.2.iv Cyclin D1/cdk4 activity lowers E2F4 mediated transactivation potential

The cyclin D1 potential regulation of E2F4 was studied next. As a first step to finding a functional outcome for the interaction, the E2F4 mediated transactivation was analyzed after transient transfection of cyclin D1 and cdk4. It is known that cyclin D1/cdk4 activity rescues p107 and p130 suppression of E2F4 mediated transactivation (Beijersbergen et al., 1995; Johnson, 1995; Xiao et al., 1996). D-type cyclin/cdk4 activity can also induce gene expression from the E2F1 promoter by derepression (Johnson, 1995; Johnson et al., 1994).

A reporter plasmid possessing four tandem E2F binding sites from the E2A promoter of adenovirus fused to the chloramphenicol acetyl transferase (CAT) gene was used in an attempt to find the effect of cyclin D1/cdk4 activity directly on E2F4. The transfection efficiency was monitored by a co-transfected luciferase reporter plasmid pGL3-Control (Promega). When cyclin D1 and cdk4 were co-expressed with E2F4 in C33A

Figure 3.5 Cyclin D1/cdk4 represses E2F4 mediated transactivation. (A) C33A cells were co-transfected with 2ug of $E2F_4$-CAT reporter plasmid, 2ug of pGL3-Control as an internal control on their own or with 2ug of pCMVHAE2F-4 and 5ug of expression vectors carrying the proteins for cdk4wt, cyclin D1, cdk4dn, cyclin E and cdk2wt as indicated. Each transfection may also contain pcDNA3 to make up to a total of 16ug of transfected DNA. CAT activities were determined for the various transfections and the values were normalized for the corresponding luciferase activities. The results are shown as a percentage of activity from the E2F4 individual transfection. The results are averages of at least three experiments with error bars representing standard error of the mean. (B) Titrating cdk4 represses E2F4/Dp-1 mediated transactivation. C33A cells were co-transfected as indicated with 2ug of $E2F_4$-CAT reporter plasmid, 2ug of pGL3-Control as an internal control, 2ug of pCMVHAE2F-4, 2ug of pcmvDp-1 and 5ug of Rc/cycD on their own or with 2, 6 and 8ug of pCMVcdk4wt. Each transfection may also contain pcDNA3 to make up to a total of 21ug of transfected DNA. CAT activities were determined for the various transfections and the values were normalized for the corresponding luciferase activities. The results are shown as a percentage of activity in the absence of cdk4. The results are averages of at least three experiments with error bars representing standard error of the mean.

cells, an inhibitory effect on E2F4 transactivation from the E2F promoter was observed (Fig. 3.5A). With the co-expression of cyclin D1 and cdk4, the relative E2F4 mediated activity was reduced as much as 75%. The suppression was dependent on active kinase since the expression of a kinase inactive dominant negative cdk4, cdk4dn, had a negligible effect. Furthermore, the results indicated a specific kinase requirement. Cdk2 and cyclin E co-expression did not have a significant effect on the transactivation potential. The effect of cdk4 activity on E2F4 transactivation was also studied in association with its dimerization partner Dp-1 (Fig. 3.5B). This was accomplished by co-expressing E2F4, Dp-1 and cyclin D1 with increasing amounts of cdk4. With increasing cdk4 expression a downward trend was noted in the transactivation potential for E2F4/Dp-1. This confirmed the previous experiments that an active cdk4 decreased the ability of E2F4 to transactivate transcription from the promoter in a pocket protein independent manner.

3.2.v Cyclin D1/cdk4 disrupts the E2F4 DNA binding activity

Electrophoretic Mobility Shift Assay (EMSA) was used to determine whether E2F4/Dp-1 retained its DNA binding activity when co-expressed with cyclin D1 and cdk4. The decrease in transactivation potential observed from the previous experiments might be explained if the DNA binding ability of E2F4 is compromised in these assays. These studies employed E2F4-Ha and Dp-1 co-transfected C33A cellular extracts. Exogenous E2F4-Ha/Dp-1 complex formation with a ^{32}P oligonucleotide end labeled probe containing the consensus E2F binding motif was first assessed in nuclear extracts (Fig. 3.6). A banding pattern consistent with exogenous E2F4-Ha/Dp-1 complex formation with DNA is apparent (Fig. 3.6 lane 1 compared with lane 7). The specificity of complex formation was determined by the addition of unlabeled specific probe versus mutant unlabeled probe. With the addition of specific unlabeled probe the DNA binding complex was disrupted as compared to when the mutant probe was added (Fig. 3.6 lane 2 compared with lane 3). The

Figure 3.6 Nuclear extracts of exogenously expressed E2F4 and Dp-1 form a complex with DNA. DNA binding activity from nuclear extracts of C33A cells untransfected (lanes7,8,9,10,11,12) or transiently transfected with 5ug each of pCMVHAE2F-4 and pcmvDp-1 (lanes1,2,3,4,5,6) were tested in electrophoretic mobility shift assays (EMSA). 1ul of E2F4 (Santa Cruz) or Ram antibody and/or 75ng of mutant (mt. comp.) and specific competitor (sp. comp.) were employed.

Figure 3.7 The effect of co-expressing cyclin D1/cdk4 to the E2F4/DP-1 DNA binding activity. (A) Nuclear extracts co-expressing cyclin D1/cdk4 lose E2F4/Dp-1 DNA binding activity. C33A cells were transiently transfected with 5ug each of pcmvDp-1 and pCMVHAE2F-4 alone (lanes 1-3) or together with 10ug of pCMVcdk4wt and 5ug of Rc/cycD (lanes 4-6), 10ug of pCMVcdk4dn (lanes 7-9) and 5ug pcmvCycE and 10ug of pCMVcdk2wt (lanes 10-12). The E2F4 DNA binding activity was assayed by EMSA employing 1ul of E2F4 (Santa Cruz) and Ram antibodies. (B) Cytoplasmic extracts co-expressing cyclin D1/cdk4 retain E2F4/Dp-1 DNA binding activity. The cytoplasmic extracts of the transfections in (A) were compared to the nuclear extracts for the E2F4 DNA binding activity assayed by EMSA. C-denotes cytoplasmic extract and N-denotes nuclear extract. (C) E2F4 and Dp-1 are expressed in the nuclear and cytoplasmic fractions. The cytoplasmic and nuclear extracts from (A) and (B) were separated by electrophoresis on SDS-7.5% polyacrylamide gels, blotted and probed for E2F4 or Dp-1 (Santa Cruz).

DNA binding complex was also assessed in terms of the components present by the use of specific and non-specific antibodies. With the addition of an anti-E2F4 antibody the protein-DNA complex was blocked from forming (Fig. 3.6 lane 4). This contrasted with the addition of non-specific Ram antibody (Fig. 3.6 lane 6). These results indicate that the endogenous E2F4 and Dp-1 proteins do not form discernible DNA-protein complexes nor did the transfection of either protein on its own in these cells (data not shown). In addition, no higher order DNA binding complexes containing E2F4-Ha and Dp-1 were visible.

E2F4 is present in both nuclear and cytoplasmic fractions, but cyclin D1 activated cdk4 is present in the nucleus (Diehl and Sherr, 1997; Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). Thus, the ability of E2F4-Ha/Dp-1 to bind DNA was assessed in the presence of cyclin D1/cdk4 using nuclear and cytoplasmic extracts from cells transfected by these components. E2F4-Ha/Dp-1 complex interaction with DNA was undetectable in nuclear extracts with co-expression of the kinase and its cyclin partner (Fig. 3.7A, lanes 1-3 compared with lanes 4-6). This was specific for a cdk4 kinase activity as cyclin E/cdk2 or cdk4dn when co-expressed were unable to inhibit DNA complex formation (Fig. 3.7A, lanes 7-12). The cdk4 inactive cytoplasmic fractions retained their DNA binding ability (Fig. 3.7B, lanes 5,6 compared with lanes 7,8). E2F4 and Dp-1 are readily detected in nuclear and cytoplasmic fractions as disclosed by Western blotting (Fig. 3.7C). The lack of DNA binding activity did not correspond to a decrease in the stability of the E2F and Dp-1 proteins or changes in their subcellular localization. These results suggest that the E2F-4/Dp-1 heterodimer no longer binds DNA in the presence of cyclin D1 and cdk4.

3.2.vi Cyclin D1 and cdk4 activity results in E2F4 hyperphosphorylation

The potential of E2F4 to be phosphorylated by cdk4 directly was tested. Presently, the only *bona fide* targets for cdk4 phosphorylation are the pocket proteins

Figure 3.8 Cdk4 phosphorylates E2F4 *in vivo*. (A) C33A cells were untransfected (lane1) and transiently transfected by pCMVHaE2F-4 on its own (lane2) or together with pCMVcdk4 (lane3). Protein from the cell lysates were immunoprecipitated for Ha (12CA5), separated by electrophoresis on an SDS-7.5% polyacrylamide gel, blotted and probed for E2F4 (Santa Cruz). * denotes increased concentration of slowest migrating form. (B) Baculovirus expressed E2F4 is phosphorylated *in vitro* by cyclin D1/cdk4 activity. SF9 cells were infected by the indicated baculovirus expressing proteins. Cells were immunoprecipitated by a polyclonal cyclin D1 antibody and subjected to an *in vitro* kinase reaction with ^{32}P-ATP. SDS release buffer was added to the cyclin D1 associated proteins. The associated proteins were re-immunoprecipitated by the Ha antibody 12CA5. (C) Overexpressed E2F4 is phosphorylated *in vitro* by a cyclin D1 endogenous catalytic subunit. C33A cells were transfected by the cmv vectors containing the indicated proteins. Equal concentrations of the transfected lysates were subjected to the same treatment as in Figure 3.8B above.

(Beijersbergen et al., 1995; Dowdy et al., 1993; Ewen et al., 1993; Hatakeyama et al., 1994; Hinds et al., 1992; Li et al., 1993; Lundberg and Weinberg, 1998; Matsushime et al., 1992; Suzuki-Takahashi et al., 1995). In these experiments, co-expressed E2F4 with cdk4 resulted in an increase in concentration of the slowest migrating form detected by Western blotting of immunoprecipitated E2F4-Ha (Fig. 3.8A). This band represents the most actively phosphorylated form of E2F4 as deduced from experiments employing lambda phosphatase treatment of transfected lysates (Fig. 3.10B). The co-expression of cyclin D1 with cdk4 was less efficient in capturing the uppermost form in Western blots. The *in vitro* potential of cyclin D1/cdk4 to phosphorylate E2F4 in SF9 viral protein expressing cellular lysates was also tested. Cells were infected with virus expressing E2F4-Ha in the presence or absence of virus expressing cyclin D1 and cdk4 (Fig. 3.8B). Lysates of the infected cells were immunoprecipitated for cyclin D1 (DSC-6) and subjected to an *in vitro* kinase assay with ^{32}P-ATP. The presence of a potentially phosphorylated E2F4 in a cyclin D1 immune complex was assessed by a re-immunoprecipitation assay. This was performed to minimize the presence of phosphorylated background proteins. SDS release buffer was added to the kinase reaction to disrupt protein-protein interactions and the contents were re-immunoprecipitated with the Ha antibody. In the presence of cyclin D1 and cdk4, E2F4-Ha was phosphorylated and thus represented a member of the cyclin D1 immune complex (Fig. 3.8B).

The ability of cyclin D1 to phosphorylate E2F4 in transfected C33A cells via an endogenous catalytic partner also was assessed. In these experiments, cyclin D1 and E2F4 were transfected on their own or together. As with the baculovirus infections above, the transfected cell lysate was immunoprecipitated with cyclin D1, an *in vitro* kinase reaction was performed and the contents of the immune complex re-immunoprecipitated with the anti-Ha antibody 12CA5 (Fig. 3.8C). This experiment revealed that an endogenous catalytic partner of cyclin D1 was able to phosphorylate E2F4-Ha *in vitro* and corroborated

Figure 3.9 Dp-1 preferably interacts with certain phosphorylated forms of E2F4. (A) C33A cells were co-transfected with cmv based plasmids expressing the proteins E2F4-Ha and Dp-1. Transfected cells were radiolabeled with ^{32}P ortho-phosphate. 4hr. post labeling, transfected cells were lysed and proteins from cellular lysates immunoprecipitated by anti-Ha 12CA5 or Dp-1. The immunoprecipitations were loaded on a 7.5% polyacrylamide gel, electrophoresed and viewed by autoradiography. (B) The proteins corresponding to E2F4 from the gel in (A) were excised, doubly digested with trypsin and V8 protease and subjected to phosphopeptide analysis. Separation in the first dimension by thin layer electrophoresis in 1.9 pH buffer and in the second by ascending chromatography using iso-butyric acid (Lacy and Whyte, 1997). Phosphopeptide maps of the Ha immunoprecipitated E2F4 and the Dp-1 immunoprecipitated E2F4, following electrophoresis and chromatography, are shown. A and B denote two phospho-peptide forms of E2F4. F.P. denotes free phosphates.

Figure 3.10 The Ha-tagged E2F4 deletion mutants. (A) The schematic representation of full length and mutant E2F4 proteins employed to deduce the cyclin D1 binding region. Important features and functional domains with their relative amino acid positions are denoted beginning with the first methionine in E2F4. (B) The *in vivo* expression pattern of the E2F4 mutants. The expression vectors for the E2F4 Ha-tagged mutants represented in (A) were transiently transfected into C33A cells, ^{35}S met/cys-labeled lysates were immunoprecipitated by 12CA5 (Ha monoclonal antibody) with and without alkaline phosphatase treatment, separated by electrophoresis on SDS-10% polyacrylamide gels and visualized by fluorography.

the results obtained using the baculovirus system. The transfection of cdk4 was not necessary in this assay because *in vitro* phosphorylation results in readily detectable protein.

3.2.vii Phosphorylation of E2F4 regulates its interaction with Dp-1

The effect of E2F4 phosphorylation on the Dp-1/E2F4 complex was determined by phosphopeptide mapping. C33A cells were transfected with E2F4 and Dp-1. The transfected cells were labeled with ^{32}P ortho-phosphate and proteins from cellular lysates were immunoprecipitated for E2F4 with anti-Ha or for Dp-1 (Fig. 3.9A). Unlike E2F4, Dp-1 did not appear to be phosphorylated. The E2F4 fraction that co-immunoprecipitated with Dp-1 and the total E2F4 pool immunoprecipitated with Ha were protease digested and separated by two dimensional chromatography (Fig. 3.9B). The results showed the Dp-1 potential to interact with only a subset of the E2F4 phosphopeptides (Fig. 3.9 spot A compared to spot B). The two spots to the left of A in the Dp-1 immunoprecipitated form probably are not from E2F4, as they are not present in the Ha immunoprecipitated fraction. These results suggested that certain phosphorylated forms of E2F4 cannot interact with Dp1.

3.2.viii Cyclin D1 contains two binding sites on E2F4 located at the Dp-1 Dimerization and Pocket Protein Binding domains

Deletion mutants for E2F4 were employed as a means to localize the cyclin D1 binding domain (Fig. 3.10A). These mutants contained an N-terminal Ha epitope and were expressed using CMV based vectors. The expression pattern for the deletion mutants in Figure 3.10A is visualized in Figure 3.10B. Lambda phosphatase was employed to determine whether the multiple bands observed were due to phosphorylation. The phosphatase treated mutants collapsed into one predominant fastest migrating band

suggesting that the banding pattern was not due to breakdown products or truncated protein expression (Fig. 3.10B).

The vectors for the N-terminal deletions were transiently transfected, with and without the cyclin D1 expression vector Rc/cycD, into C33A cells (Fig. 3.11A). The expressed proteins were tested for cyclin D1 binding by immunoprecipitation and Western blotting. An anti-Ha immunoprecipitation was positive for cyclin D1 only in co-expressed lysates suggesting that these N-terminal mutants interacted with cyclin D1 *in vivo* (Fig. 3.11A, lanes 6,8,20,22,24). The C-terminal deletion mutants were also shown to bind to cyclin D1 by employing the same strategy as above (Fig. 3.11B, lanes12,14,16,18). The cyclin D1 interaction with the shortest N-terminal and the C-terminal mutant, 179NT and 176CT respectively, implied that there are two different binding sites for cyclin D1 on E2F4 (Fig. 3.11A, lane 22 and Fig. 3.11B, lane18). Thus, the strategy employed to find both binding sites made use of mutants with both ends of the molecule deleted. In this manner the mutant 128NT/382CT was shown to bind to cyclin D1 whereas 179NT/382CT did not (Fig. 3.11C, lane12 compared with lane 14). These results suggested that an N-terminal region represented by the Dp-1 dimerization domain and the C-terminal domain on E2F4 which interacts with pocket proteins are the cyclin D1 binding sites. Experiments with ^{35}S met/cys labeled lysates, from transfections as performed in Fig. 3.11C, supported the previous findings (Fig. 3.11D). In these experiments, the mutant 179/382CT was confirmed not to co-immunoprecipitate with cyclin D1, as compared to the mutant 128/382CT (Fig. 3.11D, lanes 6,10 compared to lanes 12,14). The interaction of cyclin D1 at the Dp-1 dimerization domain suggests that cyclin D1 might be interacting with bound Dp-1 at this site. This is consistent with the idea that Dp-1 might be bridging the interaction with E2F1, E2F2 and E2F3.

Figure 3.11 E2F4 has two cyclin D1 binding domains. (A) Cyclin D1 binds *in vivo* to the N-terminal E2F4 deletion mutants. 5ug of the expression vectors for the N-terminal E2F4 Ha- tagged deletion mutants were transiently transfected on their own or with 5ug of Rc/cycD into C33A cells. Proteins from the cell lysates were immunoprecipitated as indicated, separated by electrophoresis on SDS-10% polyacrylamide gels, blotted and probed for cyclin D1 (Upstate Biotechnology). (B) Cyclin D1 binds *in vivo* to the C-terminal E2F4 deletion mutants. 5ug of the expression vectors for the C-terminal E2F4 Ha-tagged deletion mutants were transiently transfected on their own or with 5ug of Rc/cycD into C33A cells. Proteins from the cell lysates were immunoprecipitated as indicated, separated by electrophoresis on SDS-10% polyacrylamide gels, blotted and probed for cyclin D1 (Upstate Biotechnology). (C) Cyclin D1 does not bind an E2F4 Ha-tagged deletion mutant 179NT\382CT minus the Dp-1 Dimerization and the Pocket Protein Binding domains. C33A cells were transiently transfected with 5ug of the Ha-tagged expression vectors for the indicated E2F4 deletion mutants on their own or with 5ug of Rc/cycD. Proteins from the cell lysates for each transfection were immunoprecipitated as indicated, separated by electrophoresis on SDS- 10% polyacrylamide gels, blotted and probed for cyclin D1(Upstate Biotechnology). (D) The transfections and immunoprecipitations in (C) were repeated but employing ^{35}S met/cys labeled lysates and visualized by fluorography.

3.2.ix Cyclin D1 and D3 interacting domains on E2F4 are different

The cyclin D3 binding domain was mapped on E2F4. This was accomplished by employing the cmv based Ha-tagged E2F4 deletion mutants in binding studies with co-expressed cyclin D3. The same technique was applied for mapping the cyclin D1 binding domain except anti-cyclin D3 was utilized in Western blotting. Western blotting for cyclin D3 revealed that all the N-terminal E2F4 deletions interacted with cyclin D3 (Fig. 3.12A 10,12,18,20). However, the C-terminal deletion 305CT bound to cyclin D3 whereas 202CT failed to interact (Fig. 3.12B lane 12 compared with lane 16). The results demonstrate that the binding domain for cyclin D3 is different than for cyclin D1. The area of E2F4 required to bind cyclin D3 is localized between amino acid residues 305 and 179. This region denotes a unique domain of E2F4 that is not present in the other E2F family members.

3.2.x The DNA binding activity of 382CT is unaffected by co-expression of Cyclin D1 and cdk4

To determine whether the cyclin D1 site contributes to the loss in DNA binding potential the C-terminal 382CT mutant was tested in EMSA. The 382CT mutant when co-expressed with Dp-1 was capable of binding to the ^{32}P endlabeled oligonucleotide probe in nuclear extracts of transfected cells (Fig. 3.13A). An anti-E2F4 antibody (c-20 Santa Cruz) had no effect in disrupting the complex because its epitope on E2F4 is deleted. Specific binding was confirmed by the anti-Ha antibody which disrupted the complex. Nuclear extracts of cells transfected with cyclin D1 and cdk4 co-expressed with 382CT and Dp-1 revealed that DNA complex formation was unaffected. This result contrasted with our previous results with full length E2F4. (Fig. 3.13A compared to Fig. 3.7A). Active cdk4 is presumed from Western blots that depict the presence of cyclin D1 and cdk4 in

nuclear extracts (Fig. 3.13B). These results imply that the cyclin D1 C-terminal site is necessary for the regulation of E2F4 DNA binding activity by cyclin D1 targeted cdk4 phosphorylation. E2F4 with deletion of the N-terminal cyclin D1 binding domain was not used in these studies because this corresponded to the Dp-1 dimerization domain. Dp-1 is a necessary component for E2F4 to bind DNA in the EMSA method employed in these experiments.

Figure 3.12 The *in vivo* cyclin D3 interaction maps to a region between amino acid 179 and 305 of E2F4. (A) Cyclin D3 *in vivo* potential interactions with the N-terminal E2F4 deletion mutants. C33A cells were untransfected (lane 7,8) or transiently transfected by plasmids expressing cyclin D3 (lane 1,2), 44NT (lane 3,4), 84NT (lane 5,6), 128NT (lane 13,14) and 179NT (lane 15,16) and cyclin D3 together with 44NT (lane 9,10), 84NT (lane 11,12), 128NT (lane 17,18), 179NT (lane 19,20) or E2F4-Ha (21,22). Proteins from cellular lysates were immunoprecipitated for cyclin D3 (C-16) and Ha-tagged proteins (12CA5), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for cyclin D3 (C-16). (B) Cyclin D3 *in vivo* potential interactions with the C-terminal E2F4 deletion mutants. C33A cells were transiently transfected by plasmids expressing cyclin D3 (lane 1,2), 305CT (lane 3,4), 382CT (lane 5,6), 202CT (lane 7,8) and 176CT (lane 9,10) and cyclin D3 together with 305CT (lane 11,12), 382CT (lane 13,14), 202CT (lane 15,16) and 176CT (lane 17,18). Proteins from cellular lysates were immunoprecipitated for cyclin D3 (C-16) and Ha-tagged proteins (12CA5), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for cyclin D3 (C-16).

Figure 3.13 The DNA binding activity of 382CT/Dp-1 is not affected by cyclin D1/cdk4. (A) DNA binding activity from nuclear extracts of C33A cells transiently transfected with 5ug each of pcmv382CT and pcmvDp-1 alone (lanes 1-5) or together with 10ug of Rc/cycD and 5ug pCMVcdk4wt (lanes 6-10) were tested for 382CT DNA binding activity employing EMSA with 1ul of the indicated antibodies. (B) Cyclin D1 and cdk4 are co-expressed with 382CT/Dp-1. The nuclear and cytoplasmic fractions from (A) were separated on SDS-10% polyacrylamide gels, blotted and probed for cyclin D1 and cdk4. N denotes nuclear fraction and C denotes cytoplasmic fractions.

3.3 Conclusion

The experiments in this chapter describe a novel regulation of E2F activity based on the direct interaction of E2F4 with cyclin D1. Evidence provided indicates that E2F4 can bind directly cyclin D family members *in vitro* and *in vivo*. Furthermore, the effect of binding correlates to targeted phosphorylation by activated cdk4 *in vivo*. The kinase activity on E2F4 results in the disruption of its DNA binding potential by eliminating the ability to bind to its heterodimerization partner Dp-1.

Results relying on infected insect cell lysate suggest that E2F4, E2F5 and Dp-1 binding to D-type cyclins is direct despite the potential availability of host cell cycle proteins to bridge the interaction. The contributions of host cell proteins are not evident in Western blots for cyclin D1 and cyclin D3 of singly infected control lysates. The *in vivo* binding data demonstrates that E2F1, E2F2, and E2F3 are less efficient in binding than E2F4, E2F5 and Dp-1 to cyclin D1. On the other hand, cyclin D3 does not interact *in vivo* with E2F1, E2F2 and E2F3, but can bind with E2F4, E2F5 and poorly with Dp-1. These results are consistent with Dp-1 facilitating the interaction of cyclin D1 with E2F1, E2F2 or E2F3. This is corroborated by the binding region on E2F4 for cyclin D1 mapping to the Dp-1 dimerization domain. For cyclin D3, Dp-1 poorly interacts suggesting that its interaction might be facilitated by E2F4 or E2F5. This is confirmed by the cyclin D3 binding region on E2F4 that maps only to the C-terminus. Thus, Dp-1 is not available to bridge a potential interaction of cyclin D3 with E2F1, E2F2 or E2F3.

The effect of cyclin D1 interaction is to lower the transactivation potential of E2F4 through the kinase activity of the catalytic partner. This result is corroborated by three different findings presented. 1) The ability of E2F4 to be phosphorylated by the action of the interacting cyclin D1 and cdk4 kinase in baculo lysates expressing recombinant proteins or transfected cellular lysates. 2) A phosphorylated form of E2F4 that does not interact

with Dp-1. 3) In EMSA E2F4/Dp-1 DNA binding activity was nullified in nuclear extracts where cdk4 is active as opposed to the cytoplasmic extracts.

Chapter 4. Mapping of the p130 regions required to interact with E2F4, E2F5 and Dp-1

4.1 Introduction

A functional characteristic of the pocket proteins is their inhibitory role in the cell cycle which is thought to occur, at least partly, through their interaction with the E2F family of transcription factors. However, the mechanism of E2F control is not completely understood. For example, it is not known how the pocket proteins act in regulating E2F function. The elucidation of the E2F interacting domains on the pocket proteins provides a starting point to analyze how control is facilitated. The E2F interaction domains on p107 and pRb are similar. pRb and p107 require the A and B domains of the pocket region for binding to E2F (Hiebert et al., 1992; Qian et al., 1992; Smith and Nevins, 1995; Zhu et al., 1995). It is hypothesized that the A and B domains provide a functional interface by which E2F can bind (Lee et al., 1998). The A/B pocket is also required to interact with the oncogenes of DNA tumour viruses containing the LxCxE motif (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). The E2F binding domain on pRb and p107 has not been deduced for any specific E2F family member. In addition, a Dp-1 potential interaction has not been ascertained for p107 and p130. As a means to begin developing a hypothesis for how p130 regulates E2F, an investigation of the regions of p130 necessary to interact with E2F4, E2F5 and Dp-1 was initiated. In addition, the potential of Dp-1 to complex directly with p130 was also studied.

Figure 4.1 Expression pattern of p130 deletion mutants. (A) Schematic representation of full length and mutant p130 proteins. The A, B and Spacer domains with their amino acid positions are denoted. (B) C33A cells were untransfected or transiently transfected by the indicated N-terminal p130 deleted mutants. Proteins from the transfected cellular lysate were immunoprecipitated for p130 (ctp130), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for p130 (ctp130). (C) C33A cells were untransfected or transiently transfected by the indicated C-terminal p130 deleted mutants. Cells were metabolically labeled with ^{35}S-methionine/cysteine, immunoprecipitated for p130 (ctp130), separated by electrophoresis on a 7.5% polyacrylamide gel and visualized by fluorography. MW denotes the size of molecular weight markers.

4.2 Results

4.2.i Endogenous E2F4 interacts with overexpressed N-terminal p130 deletions

Mapping of the binding site for E2F4, E2F5 and Dp-1 on p130 relied on an *in vivo* overexpression system of CMV based plasmids expressing p130 proteins with amino acid deletions. To determine whether the A or B domains of p130 were necessary to bind E2F, a series of progressive N-terminal deletions spanning the A and spacer domains and a second series of C-terminal deletions encompassing the B domain were generated (Fig. 4.1). The expression pattern of the N-terminal and C-terminal deletion mutants were examined in Figure 4.1B and C. The N-terminal plasmids expressing p130 deletion mutants were transiently transfected into C33A cells immunoprecipitated and Western blotted for p130 (Fig.4.1B). The C-terminal mutants 932N and 835N, along with 372C-BN were also transiently transfected into C33A cells. These were visualized by metabolic labeling with ^{35}S-methionine/cysteine of transfected cells immunoprecipitated for p130 (Fig. 4.1C). 932N and 835N did not express to the same efficiency as the other p130 mutants. The reason for this is not known but it might reflect a loss of structural stability.

The potential of the N-terminal mutants to bind endogenous E2F4 from C33A cells was tested by Western blotting techniques. C33A cells contain detectable levels of E2F4. In these experiments C33A cells were transiently transfected with the N-terminal deletion mutants 372C, 542C and 602C. Proteins from cellular lysates were immunoprecipitated for p130 and E2F4 in parallel and assayed for binding by probing the Western blot for p130 (Fig. 4.2). All the mutants employed interacted with E2F4 (Fig. 4.2 lanes 6,8,10). In addition, the ability of endogenous E2F4 and p130 to bind was also apparent (Fig. 4.2 lane 1and2). The results also indicated that underphosphorylated forms of p130 mutants preferentially interacted with E2F4 (Fig. 4.2 lanes 6,8,10 compared with 5,7,9).

Figure 4.2 p130 N-terminal mutants interact with endogenous E2F4. C33A cells were untransfected (Control) or transiently transfected by the indicated N-terminal p130 deleted mutants. Proteins from the transfected and untransfected cellular lysate were immunoprecipitated for p130 (ctp130) and E2F4 (C20) in parallel. Immunoprecipitations were separated by electrophoresis on a 7.5% polyacrylamide gel, blotted and probed for p130 (C20).

4.2.ii E2F5 interacts with p130 mutants 820C and (-sp)p130

The concentration of cellular lysate employed in the previous experiment caused a high background. Furthermore, E2F5 levels are not detected in C33A cells. Therefore, in an attempt to obtain a sharper image and minimize the contribution of endogenous proteins, Ha-tagged versions of Dp-1, E2F4 and E2F5 were used in subsequent studies. The E2F5 binding potential with various N-terminal and spacer mutants of p130 was evaluated next. This was accomplished in C33A cells transiently transfected by E2F5-Ha or E2F5-Ha co-expressed with p130, 372C, 820C and (-sp)p130. Transfected cells were immunoprecipitated for Ha-tagged E2F5 and for p130, and the immunoprecipitations were analyzed for the presence of p130 by Western blotting. Overexpressed E2F5 interacted with each of the p130 N-terminal mutants (Fig. 4.3A,B lane 4). In addition, as with E2F4, the binding of E2F5 to p130 was not dependent on the presence of an intact spacer domain (Fig. 4.3B lane 6). The interaction with endogenous p130 is not apparent in these studies as less lysate was employed.

4.2.iii Dp-1 interacts *in vivo* with N-terminal p130 mutation 820C

Dp-1 is highly homologous to the E2F family at the heterodimerization domain and contains an acid rich C-terminus that might facilitate binding with pocket proteins (Bandara et al., 1993; Shan et al., 1996). Dp-1 was unable to complex with 372C and p130 employing the baculo virus system (results not shown). However, *in vivo* the potential of Dp-1 to bind to p130 and p130 mutants provided the opposite result. This was accomplished by transient transfections of C33A cells expressing p130, 372C and 820C co-expressed with Ha-tagged Dp-1. p130 was observed to bind to Dp-1, the N-terminal mutants 372C and 820C also interacted (Fig. 4.4 lane 2,4,6). However, the inability of the interaction to take place in the baculovirus system raises the possibility that the interaction with p130 might be bridged by endogenous E2F-like proteins.

Figure 4.3 p130 missing N-terminal sequences up to amino acid 820, and (-sp)p130 can complex with E2F5. (A) C33A cells were untransfected or transiently transfected by plasmids expressing E2F5-Ha or E2F5-Ha together with p130 or 820C. Proteins from cellular lysates were immunoprecipitated for p130 (ctp130) and Ha-tagged E2F5 (12CA5), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for p130 (C20). (B) C33A cells were transfected by plasmids expressing E2F5-Ha together with p130, 372C or (-sp)p130. Proteins from cellular lysate were immunoprecipitated for p130 (ctp130) and Ha-tagged E2F5 (12CA5), separated by electrophoresis on a 7.5% polyacrylamide gel, blotted and probed for p130 (C20).

Figure 4.4 p130 missing N -terminal sequences up to amino acid 820 can complex with Dp-1. C33A cells were untransfected or transiently transfected by plasmids expressing Dp-1-Ha or Dp-1-Ha together with p130, 372C or 820C. Proteins from cellular lysates were immunoprecipitated for p130 (ctp130) and Ha-tagged Dp-1 (12CA5), separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for p130 (C20).

4.2.iv E2F5 and Dp-1 interact with a cyclin A- and E-binding defective mutant of p130, 372C-BN

A p130 mutant that is unable to bind to cyclins A and E also was evaluated for binding to E2F5 and Dp-1. 372C-BN lacks the cyclin binding domains at the spacer and N-terminal regions (Castano et al., 1998; Lacy and Whyte, 1997; Woo et al., 1997). C33A cells, transiently transfected with 372C-BN or 372C-BN together with E2F5 or Dp-1, were metabolically labeled with ^{35}S-methionine/cysteine. A Western blot for 372C-BN was not feasible as its apparent molecular weight of 66kd, falls within the heavy chain immunoglobulin signal (Fig. 4.1C). Labeled lysate was immunoprecipitated in parallel for p130 and Ha-tagged E2F5 or Dp-1. E2F5 and Dp-1 formed a complex with 372C-BN as indicated by co-immunoprecipitation of both co-expressed proteins (Fig. 4.5 lane 3,4,5,6). E2F4 was not tested for binding to 372C-BN, as both proteins migrate at approximately the same location in polyacrylamide gels and cannot be distinguished in co-immunoprecipitation experiments.

4.2.v E2F4, E2F5 and Dp-1 interact with the p130 N-terminal mutant 820C

The 820C mutant lacks domain A and the spacer region and represents the extent of the N-terminal deletions that were constructed. The ability of this protein to interact with E2F4, E2F5 and Dp-1 was compared. This was performed by transfecting C33A cells with 820C or 820C in combination with E2F4, E2F5 and Dp-1. Proteins from transfected lysates were immunoprecipitated for p130 and Ha-tagged E2F4, E2F5 and Dp-1 and probed for p130 in a Western blot (Fig. 4.6). 820C interacted with each of the transcription factors, but the interaction with E2F4 was significantly diminished as compared to E2F5 and Dp-1 (Fig. 4.6 lane 6 compared to lanes 8 and 10). These results

Figure 4.5 Dp-1 and E2F5 can interact with 372C-BN. C33A cells were untransfected or transiently transfected by plasmids expressing 372C-BN and 372C-BN together with Dp-1-Ha or E2F5-Ha. Cells were metabolically labeled with ^{35}S-methionine/cysteine, immunoprecipitated for p130 (ctp130) and Ha-tagged Dp-1 and E2F5, separated by electrophoresis on a 7.5% polyacrylamide gel and visualized by fluorography.

Figure 4.6 Comparison of the E2F4, E2F5 and Dp-1 interaction with 820C. C33A cells were untransfected or transiently transfected by plasmids expressing 820C and 820C co-expressed with E2F4-Ha, E2F5-Ha or Dp-1-Ha. Proteins from cellular lysates were immunoprecipitated for p130 (ctp130) and Ha-tagged proteins, separated by electrophoresis on a 10% polyacrylamide gel, blotted and probed for p130 (C20).

together with the results derived with (-sp)p130 suggest that E2F4, E2F5 and perhaps Dp-1 interact at the same domain on p130. In the case of E2F4, amino acids between 697 and 820 in the spacer of p130 might confer a more stable interaction.

4.2.vi. E2F4, E2F5 and Dp-1 interact with the p130 mutant 932N, but not with 835N

To complete the mapping studies, the extent of C-terminal sequences required for binding was examined by enlisting the 835N and 932N proteins (Fig. 4.7A,B,C). 835N contains domain A and the spacer element and part of domain B whereas 932N contains an intact A/B pocket and spacer element. Transient transfections of C33A cells with plasmids expressing these proteins on their own or with E2F4, E2F5 and Dp-1 were initiated. Lysates from the transfections were immunoprecipitated in parallel for Ha or p130 and probed for p130 in Western blots. The results indicated that the 932N construct was capable of interacting with E2F4, E2F5 and Dp-1 (Fig. 4.7A,B,C lane 11). However, 835N did not interact with these transcription factors (Fig. 4.7A,B lane 13 and Fig. 4.7C lane 14).

Figure 4.7 p130 missing C-terminal sequences up to amino acid 932, but not 835, can complex with E2F4, E2F5 and Dp-1. (A) C33A cells were transfected by plasmids expressing E2F4-Ha, p130, 932N and 835N and E2F4-Ha co-expressed with p130, 932N or 835N. Proteins from cellular lysates were immunoprecipitated for p130 (ntp130) and Ha-tagged E2F4 (12CA5), separated by electrophoresis on a 7.5% polyacrylamide gel, blotted and probed for p130 (Z83). (B) and (C) The same transfections and manipulations as in (A) but E2F4-Ha was substituted by E2F5-Ha in (B) and DP-1-Ha in (C).

4.3 Conclusion

The *in vivo* mapping studies, employing N-terminal and C-terminal deletion mutants, describe a region between 835 and 932 amino acid residues on p130 for E2F4, E2F5 and perhaps Dp-1 binding. The region denotes a portion of the B domain that is necessary for the interaction. This finding reveals that the pocket proteins do not interact with similar regions, pRb and p107 require an intact A and B domain (Hiebert et al., 1992; Qian et al., 1992; Smith and Nevins, 1995; Zhu et al., 1995). This might be a reflection of p130 protein stability due to its greater size as compared to p107 and pRb. In addition, the cdk2 phosphorylation of p130 is not a pre-requisite for binding as p130 mutants that lack the ability to interact with cyclins can continue to bind to E2F4, E2F5 and Dp-1. This is emphasized by the E2F4 preferential interaction with the under phosphorylated p130 mutants.

Dp-1 was found to interact *in vitro* with pRb but, nonetheless does not interact in the baculovirus system with p130. The differences might be due to the different *in vitro* systems employed by Bandara et al, a GST-pRB fusion protein interacting with an *in vitro* translated Dp-1 protein (Bandara et al., 1993). However, Dp-1 is capable of interacting with p130 *in vivo* at the same sites as E2F4 and E2F5. The interaction might be due to endogenous proteins bridging the interaction. If the binding is direct then one possibility is that the acidic C-terminal portion of Dp-1 might have structural similarity with the E2F family of proteins in their acidic rich pocket protein binding domains (Shan et al., 1996).

Chapter 5. Cyclin D/cdk4 activity abrogates the p130-E2F4 interaction

5.1 Introduction

Further studies were initiated to investigate the mechanism of E2F4 control by p130 and D-type cyclin/ cdk4 activity. The direct interaction with D-type cyclin cdk4 activity on E2F4 causes a reduction in the E2F4 transcriptional activity (Chapter 3). D-type cyclin cdk4 activity has been implicated in the rescue of p107 mediated suppression of E2F4 transactivation in transient transfection assays (Beijersbergen et al., 1995). The manner by which this is accomplished is the disruption of the p107/E2F4 complex. For p130, cyclin D3/cdk4 activity has been correlated to the breakup of a complex containing E2F4 in BALB/c 3T3 fibroblasts (Dong et al., 1998). In addition D-type cyclin activity can rescue p130 suppression of E2F mediated activation in transient transfection assays (Johnson, 1995). Therefore, the ability of direct cdk activity to affect the p130-E2F complex was investigated.

5.2 Results

5.2.i p130 is phosphorylated in a cell cycle dependent manner

An indication that p130 might be controlled by cdk activity is the cell cycle dependent phosphorylation of p130 (Mayol et al., 1995; Mayol et al., 1996). The cell cycle dependent phosphorylation of p130 was repeated in a time course analysis of p130 phosphorylation status in BALB/c cells (Fig. 5.1). In this experiment, BALB/c cells were serum starved for 36 hours and allowed to re-enter the cell cycle upon addition of 10% FBS. At different time intervals after re-stimulation (not representative of a full cell cycle) an equal concentration of cellular lysate was immunoprecipitated and subsequently probed

Figure 5.1 Cell cycle regulated phosphorylation of p130. BALB/c cells were serum starved for 36 hr. and re-stimulated to enter the cell cycle by 10% FBS. At the indicated times after addition of serum equal concentrations of cellular lysate were immunoprecipitated for p130 (ctp130). The immunoprecipitations were loaded on to a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (Z83).

Figure 5.2 Cyclin/cdk activity promotes the phosphorylation of p130. (A) C33A cells were transiently transfected with plasmids expressing p130 (lane 1,2) or p130 co-expressed with cyclin D1/cdk4 (lane 3,4), cyclin A/cdk2 (lane 5,6) or cyclin E/cdk2 (lane 7,8). Cellular lysate from each transfection was halved and immunoprecipitated in duplicate for p130 (ctp130). One of the immunoprecipitations of each set was subjected to lambda phosphatase treatment (lane 2,4,6,8). The treated and untreated immunoprecipitations were loaded on to a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (C20).

for p130. The results implied that there are several forms of p130 due to differential phosphorylation in a cell cycle dependent manner and, in accord with published reports, the level of p130 expression is reduced at 9hr., approximating the timing of mid-G1 (Muller et al., 1997).

Cdks frequently are involved in cell cycle dependent phosphorylation events. Experiments were performed to determine whether cdks were involved in the phosphorylation of p130. C33A cells were transiently transfected with plasmids expressing p130 or p130 co-expressed with cyclin A/cdk2, cyclin E/cdk2 or cyclin D1/cdk4 (Fig. 5.2A). Immunoprecipitated p130 from transfected lysate revealed that a mobility shift to a slower migrating form occurred in the presence of all three cyclin/cdk combinations (Fig. 5.2 lane 1 compared to lane 3,5,7). Furthermore, the shift to a slower migrating form was due to phosphorylation. Phosphatase treatment resulted in a faster gel migrating form, identical to the outcome without co-expression of the cyclin/cdks (Fig. 5.2A lane 2 compared with lane 4,6,8).

5.2.ii Cyclin D1/cdk4 activity is sufficient to abrogate the E2F4 or E2F5 interaction with p130

The effect of cyclin/cdk activity on the p130 /E2F4 interaction was analyzed. As above, the cyclin/cdk combinations were transfected into C33A cells with p130 and E2F4 or E2F5. The interaction of p130 and the E2Fs were monitored by immunoprecipitating for Ha-tagged E2F4 or E2F5 and probing the immuno blots for p130 (Fig. 5.3A,B). The association of E2F4 and E2F5 with p130 was disrupted when cdk4 and cyclin D1 were co-expressed (Fig. 5.3A,B lane 4). The co-expression of cyclin A or E and cdk2 had no effect on E2F-p130 complex formation. (Fig. 5.3A,B lanes 6,8). Furthermore, the

Figure 5.3 Active cyclin D1/cdk4 disrupts the p130 interaction with E2F4 and E2F5. (A) C33A cells were untransfected (lane 13,14) or transiently transfected with plasmids expressing p130 and E2F4-Ha (lane1,2) or p130 and E2F4-Ha together with cyclinD1 and Cdk4 (lane 3,4), cyclin A (lane 9,10), cyclin A and cdk2 (lane 5,6), cyclin E (lane11,12) or cyclin E and cdk2 (lane 7,8). Proteins from the cellular lysate was immunoprecipitated in parallel for p130 (ctp130) and Ha-tagged E2F4. The immunoprecipitations were electrophoresed on a 7.5% polyacrylamide gel, blotted and probed for p130 (C20). (B) The same transfections and experimental methods as in (A) were performed except E2F4-Ha was replaced by E2F5-Ha and immunoprecipitations with Ha were for E2F5.

Figure 5.4 Cyclin D1 and D3 activated kinase disrupts the untransfected E2F4 complex with p130. (A) C33A cells were transiently transfected with plasmids expressing p130 (lane 1,2) and p130 together with cyclin A and cdk2 (lane 3,4), cyclin E and cdk2 (lane5,6), cyclin D3 and cdk4 (lane 7,8) or cyclin D1 and cdk4 (lane 9,10). Proteins from transfected cellular lysates were immunoprecipitated in parallel for p130 (ctp130) and E2F4 (C20). The immunoprecipitations were loaded onto a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (C20).

mobility of p130 in the presence of cyclin D1/cdk4 appeared to be slower than that for cyclin A or E with cdk2 (Fig. 5.3A,B lane 3 compared to lanes 5,7). These results suggested that phosphorylation of p130 by cdk4 activity, and not cdk2, contributed to break up the bound complexes.

The effect of various cyclin/cdk combinations on complex formation between endogenous E2F4 and overexpressed p130 also was assessed. C33A cells were transfected with p130 in the presence or absence of cyclin A or E and cdk2 and cyclin D1 or D3 and cdk4. A Western blot probed for p130 was performed on the immunoprecipitations for E2F4 and p130. As with overexpressed Ha-tagged E2F4 and E2F5, the endogenous E2F4 did not interact with p130 in the presence of co-expressed cyclin D1 or cyclin D3 with cdk4 (Fig. 5.4 lane 8,10). Cyclin A/cdk2 and cyclin E/cdk2 had no apparent effect on the interaction (Fig. 5.4 lane 4,6). Taken together with the previous results this experiment proposes that D-type cyclin phosphorylation of p130 causes a disruption in the interaction with E2Fs.

The next experiment was conducted to exclude the possibility that a direct cyclin D1 interaction with p130 or E2F4 prevented complex formation. For this purpose, transient transfections with p130 and E2F4 co-expressed with cyclin D1 in the presence or absence of cdk4 were performed. The transfections were immunoprecipitated for Ha-tagged E2F4 and p130 in parallel and Western blotted for p130. As before the abrogation of binding occurred only when the cyclin D1 catalytic partner was co-expressed (Fig. 5.5 compare lane 7 with lane 5).

The ability to affect the p130/E2F4 interaction *in vivo* was further assessed in U7 cells, a stable U2OS cell line derivative overexpressing p130. Overexpressed p130 in U7 cells is in a hyperphosphorylated state due to the enhanced activity of D-type cyclins and

Figure 5.5 Cyclin D1 does not compete with E2F4 for p130. (A) C33A cells were untransfected (lane 1,2) or transiently transfected with plasmids expressing p130 and E2F4-Ha (lane 3,4) or p130 and E2F4 together with cyclin D1 (lane5,6) or cyclin D1 and cdk4 (lane 7,8). Proteins from transfected lysates were immunoprecipitated in parallel for p130 (ctp130) and Ha-tagged E2F4. The immunoprecipitations were loaded onto a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (C20).

cdk4 caused by the mutation of p16 in the parental cell line. E2F4 interacts poorly with p130 in these cell lines. The potential increase in the p130/E2F4 complex formation was investigated by adding back p16 and cdk4dn. In this experiment U7 cells were transiently transfected with cdk4dn or increasing increments of p16. Cell lysate of transfected cells were normalized for protein content and immunoprecipitated in parallel for endogenous p130 and E2F4 and probed for p130 (Fig. 5.6). With the addition of cdk4dn there was an increase in the amount of E2F4 that interacted with p130 (Fig. 5.6 lane 2 compared with lane 4). Secondly, with increasing concentrations of p16 there was a proportional increase in the amount of p130 bound to E2F4 (Fig. 5.6 lane 6,8 compared to lane2). These results suggest that p16 and cdk4dn were able to dampen the D-type cyclin/cdk4 activity on p130 resulting in the increase of bound complexes.

5.2.iii The p130 interaction domain with cyclin D1 does not overlap with the E2F binding domain

As a first step to identify how D-type cyclin cdk4 activity disrupts the E2F interaction, the p130 binding domain for cyclin D1 was delineated. This was accomplished by employing the p130 CMV based deletion mutants 820C and the N-terminal mutants 835N and 932N (Fig. 4.1) in transient transfections of C33A cells with co-expressed cyclin D1. Proteins from the transfected cellular lysates were immunoprecipitated for cyclin D1 and p130 in parallel and probed for cyclin D1 (Fig. 5.7A,B). The 820C polypeptide interacted with cyclin D1, suggesting that N-terminal sequences on p130 up to amino acid 820 were not necessary for interaction with cyclin D1 (Fig. 5.7A lane 10). The C-terminal deleted mutants were unable to interact with cyclin D1 (Fig. 5.7B lane 8,10). Together these results indicate that the A domain and the spacer

Figure 5.6 cdk4dn and p16 promote the interaction of p130 with E2F4 in U7 cells. (A) U7 cells were untransfected (lane 1,2) or transiently transfected with plasmids expressing cdk4dn (lane 3,4) or increasing increments of p16, 10 ug (lane5,6) and 20ug (lane7,8) respectively. Proteins from cellular lysates were immunoprecipitated in parallel for p130 (ctp130) and E2F4 (C20). The immunoprecipitations were loaded onto a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (C20).

region of the pocket are not necessary for cyclin D1 binding. The sequences in the C-terminus that might include the B domain formed the cyclin D1 binding site. Thus, cyclin D1 and E2F4 interact with p130 at different sites with cyclin D1 requiring sequences downstream of amino acid 932 whereas E2F4 requires only sequences between 835 and 932 for binding (Chapter 4.2).

5.2.iv Cyclin D1 does not require the LxCxE motif to interact with the pocket proteins

Cyclin D1 is thought to interact with the Rb proteins via its LxCxE amino acid motif (Dowdy et al., 1993). This is analogous to the manner by which the DNA tumour virus transforming proteins, E1a ,Large T and E7, interact with p107 and pRb (DeCaprio et al., 1989; Jelsma et al., 1989; Jones et al., 1990; Kaelin et al., 1991; Whyte et al., 1989).

For binding to proteins with the LxCxE motif, crystallographic studies on pRb suggest that the A domain is necessary to stabilize the B domain where the actual interaction takes place (Lee et al., 1998). These results have been extended to the family members p107 and p130. The *in vivo* binding studies for cyclin D1 described in Figure 5.7A,B indicated that the p130 A domain was not necessary for binding. If this result is correct, it suggests that the LxCxE motif might not be required for cyclin D1 to complex with p130. This hypothesis was tested by *in vivo* studies employing a mutant of cyclin D1 at the LxCxE motif that replaces the amino acids LLCCE with LLGHE, designated as cyclin D1-GH (Dowdy et al., 1993). C33A cells were transiently transfected by cyclin D1-GH, pRb, p107 and p130 or cyclin D1-GH co-expressed with pRb, p107 or p130. Proteins from the transfected cellular lysates were immunoprecipitated in parallel for cyclin D1 and the transfected pocket protein and analyzed by Western blot for cyclin D1. The results demonstrated that p130 and the other pRb family members could interact with cyclin D1-GH *in vivo* (Fig. 5.8A lane 10,12,14). That pRb can interact with cyclin D1-GH is

inconsistent with published data (Dowdy et al., 1993). These investigators used fluorography to visualize interactions of co-immunoprecipitations of ^{35}S-methionine metabolically labeled transiently transfected COS1 cells. The same technique was repeated in C33A cells and it also demonstrated that the LxCxE motif is not essential for complex formation between cyclin D1 and the pocket proteins (Fig. 5.8B).

Figure 5.7 *In vivo* mapping of the p130 region required to interact with cyclin D1. (A) C33A cells were untransfected (lane 11,12) or transiently transfected with plasmids expressing cyclin D1 (lane1,2), p130 (lane 3,4) and 820C (lane 5,6) or cyclin D1 co-expressed with p130 (lane7,8) or 820C (lane 9,10). Proteins from transfected cellular lysates were immunoprecipitated in parallel for cyclin D1 (Upstate) and p130 (ctp130). The immunoprecipitations were loaded onto a 10% polyacrylamide gel, electrophoresed, blotted and probed for cyclin D1 (Upstate). (B) C33A cells were untransfected (lane 11,12) or transiently transfected with plasmids expressing cyclin D1 (lane1,2), 932N (lane 3,4) and 835N (lane 5,6) or cyclin D1 together with 932N (lane7,8) or 835N (lane 9,10). Proteins from cellular lysates were immunoprecipitated in parallel for cyclin D1 (Upstate) and p130 (ntp130). The immunoprecipitations were manipulated as in (A).

Figure 5.8 A cyclin D1 mutation in the LxCxE motif interacts with the pocket proteins. (A) C33A cells were transiently transfected with plasmids expressing cyclin D1-GH (lane1,2), pRb (lane 3,4), p107 (lane 5,6) and p130 (lane 7,8) or cyclin D1-GH together with pRb (lane 9,10), p107 (lane 11,12) or p130 (lane 13,14). Proteins from transfected cellular lysates were immunoprecipitated in parallel for cyclin D1 (DCS-G) or antibodies for the indicated pocket proteins. The immunoprecipitations were loaded onto a 10% polyacrylamide gel, electrophoresed, blotted and probed for cyclin D1 (DCS-G). (B) The same transfections as in (A) were performed but cells were metabolically labeled with ^{35}S-methionine/cysteine. Immunoprecipitations were carried out as in (A) and visualized by fluorography.

5.3 Conclusion

The results in this chapter describe the potential of p130 to be phosphorylated in a cell cycle dependent manner by the action of cyclin dependent kinases. The cyclin D/cdk4 activity on p130 resulted in the disruption of the p130-E2F4 interaction. The cyclins without their catalytic partners also were unable to break up the interaction. The potential of activated cdk4 to promote the disruption of p130-E2F4 complex is strengthened by the ability of cdk4dn and p16 to enhance the opposite effect in U7 cells. In this stable cell line p16 mutation leads to hyperphosphorylated p130 that interacts poorly with E2F4. However, when cdk4 activity was reduced by the addition of cdk4dn or p16 more E2F4 bound to p130. The implication of the cyclin D1/cdk4 activity on p130-E2F4 function is the derepression of E2F4 mediated transactivation. This is exhibited for p107-E2F4 that is correlated to the disruption of their complex formation with activated cyclin D/cdk4 (Beijersbergen et al., 1995; Xiao et al., 1996).

Cyclin D1 did not bind to p130 at the same site required to interact with E2F. The C-terminal domain of p130 after amino acid 932 is required to interact with cyclin D1. In addition, the interaction did not require the LxCxE motif that is required by the DNA tumour virus oncoproteins for efficient interaction. As this motif requires both the A and B domains for efficient binding it confirms the cyclin D1 interaction region is outside the A domain, requiring B domain amino acid sequences only.

Chapter 6. p130 mediates the cdk2 phosphorylation of E2F4

6.1 Introduction

E2F4 is differentially phosphorylated in a cell cycle dependent manner (Vairo et al., 1995). Some of the phosphorylation might be due to cdk2, as E2F4 can be in-vitro phosphorylated by kinases associated with p130 and p107 (Ginsberg et al., 1994; Vairo et al., 1995). The known associated kinases with p130 are cdk2 via the association of cyclin A and E, and cdk4/6 through the D-type cyclin family (Cobrinik et al., 1993; Hannon et al., 1993; Lacy and Whyte, 1997; Li et al., 1993). In Chapter 3 it was reported that E2F4 could be phosphorylated by activated cyclin D1/cdk4 directly. Cyclin A does not directly interact with E2F4, but cyclins A and E with their catalytic partner, cdk2, are known to bind p130 and p107 through the spacer portion of the A/B pocket domain and an N-terminal homologous region (Castano et al., 1998; Dynlacht et al., 1997; Ewen et al., 1992; Faha et al., 1992; Lacy and Whyte, 1997; Woo et al., 1997). The goal of the experiments presented in this chapter is to find whether cyclin/cdk2 combinations are involved in the phosphorylation of E2F4.

6.2 Results

6.2.i *In vivo* cdk2 phosphorylation of E2F4 does not involve direct cyclin A or E binding

As a means for determining the *in vivo* potential of cdk2 to phosphorylate E2F4, plasmids expressing Ha-tagged E2F4 solely or together with cdk2 were transiently transfected into C33A cells. Transfected and untransfected cells were metabolically labeled with ^{35}S-methionine/cysteine. Transfected E2F4 was immunoprecipitated from cell lysates by anti-Ha antibody 12CA5. In the presence of cdk2, a slower migrating

Figure 6.1 E2F4 phosphorylation can be generated by cdk2. C33A cells were untransfected (lane 3) or transfected with plasmids expressing E2F4-Ha solely (lane 1) or co-expressed with cdk2 (lane 2). The cells were labeled with ^{35}S-methionine/cysteine 40 hr. after transfections and cellular lysates, normalized for their protein content, were immunoprecipitated with 12CA5 directed against Ha-tagged E2F4. Immune complexes were run on a 7.5% polyacrylamide gel and visualized by fluorography. (B) and (C) SF9 cells were infected by the indicated viruses (top of figures) and metabolically labeled with ^{35}S-methionine/cysteine 40 hr. after infection. Cellular lysates were immunoprecipitated for cyclin A (C160) or cyclin E (168) (lanes 1,4,7,10), E2F4-Ha (12CA5) (lanes 2,5,8,11) or by a rabbit anti-mouse antibody (RAM) (lanes 3,6,9,12). The immune complexes were run on a 7.5% polyacrylamide gels and subjected to fluorography.

form of E2F4 was observed (Fig. 6.1A). These observations suggested that a shift to a slower migrating form was due to additional phosphorylation caused by the activity of transfected cdk2.

There are two settings by which the exogenous addition of cdk2 can alter the phosphorylation of E2F4. One method is that cyclin E or cyclin A, by directly binding to E2F4, targeted the phosphorylation by cdk2. The cyclin A and E targeting of substrates such as the pocket proteins is well documented. The other method is indirect requiring an adaptor molecule. In this framework, the adaptor can act as a bridge by binding the cyclins and/or cdk2 and E2F4 leading to the phosphorylation.

The direct binding by cyclin A or E was tested to differentiate the mechanism of phosphorylation, by employing an *in vitro* baculovirus system expressing recombinant proteins. Previous reports for E2F1 describe direct binding of cyclin A with its catalytic partner cdk2 (Krek et al., 1994; Krek et al., 1995; Xu et al., 1994). However, there is evidence that E2F4 cannot bind to cyclin A directly (Dynlacht et al., 1997). In this case purified recombinant GST E2F4 and GST cyclin A do not interact. Direct binding occurs when the cyclin A binding motif is transferred to E2F4. The feasibility of E2F4 to bind cyclins A and E was tested by co-immunoprecipitation. SF9 cells were infected with baculovirus expressing cyclin E, cyclin A and E2F4-Ha on their own or the cyclins together with E2F4-Ha. The cells were metabolically labeled by ^{35}S-methionine/cysteine and immunoprecipitated in parallel by cyclin A, cyclin E or Ha-tagged E2F4. If cyclin A or E interacted with E2F4 directly, the Ha antibody would be able to co-immunoprecipitate these cyclins with E2F4 and similarly E2F4 would co-immunoprecipitate with cyclin E and A antibodies. In baculovirus infected cells, several forms of E2F4 were observed due to

Figure 6.2 p130 enhances the phosphorylation of E2F4. (A) C33A cells were untransfected (lane 1) or transfected with plasmids expressing E2F4-Ha (lane 3) or E2F4-Ha in combination with cdk2dn (lane 2) or p130 (lane 4). Cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged E2F4 by 12CA5 40 hr after transfection. Immune complexes were loaded onto a 7.5% polyacrylamide gel, separated by electrophoresis, blotted and probed for E2F4 (C-20). (B) C33A cells were untransfected or transfected by plasmids expressing the indicated proteins (above figure). 40 hr. later the cellular lysate for each transfection was separated into two parts representing 20% and 80% of the total lysate. The lysates were normalized for protein content, immunoprecipitated for cdk2 and subjected to an *in vitro* kinase assay with ^{32}P-ATP. To the portion with *20%* sample buffer was added to stop the reaction. SDS release buffer was added to the 80% reaction. The resulting soluble fractions within the SDS buffer were re-immunoprecipitated for Ha-tagged E2F4 with 12CA5. The samples were separated on a 7.5% polyacrylamide gel by electrophoresis and visualized by autoradiography. (C) C33A cells were untransfected (lane 6) or transfected with plasmids expressing cdk2 (cdk2wt) (lane 2) or E2F4-Ha (lane 1) or E2F4-Ha in combination with cdk2wt (lane 3), p130 (lane 4) and dominant negative cdk2 (cdk2dn) (lane 5). Cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged E2F4 with 12CA5, subjected to an *in vitro* kinase assay with ^{32}P-ATP and loaded onto 7.5% polyacrylamide gels and separated by electrophoresis.

phosphorylation, but the co-immunoprecipitation with the cyclins was not observed in these experiments (Fig. 6.1B and 1C, lanes 7,8,9).

6.2.ii p130 can facilitate the phosphorylation of E2F4

From the previous results, the E2F4 phosphorylation by cdk2 is possible, but direct binding does not appear to occur. Therefore, a facilitator molecule might be bridging the cyclin and kinase to E2F4. The obvious choices for this role are p107 and p130 which are known to bind cyclin A and E with cdk2 and E2F4 . p107 and p130 can also harbour active cdk2 kinase activity and are phosphorylated in a cell cycle dependent manner by the action of cdks (Hauser et al., 1997; Mayol et al., 1995; Prall et al., 1998; Xiao et al., 1996; Zerfass-Thome et al., 1997).

The possibility that p130 can act to bridge the phosphorylation of E2F4 by cdk2 *in vivo* was assessed by employing over expressed proteins. The phosphorylation of E2F4 was monitored by transient transfections of C33A cells with plasmid vectors expressing E2F4-Ha on its own or co-expressed with cdk2dn or p130. Cdk2dn is the kinase inactive version of cdk2wt that cannot transfer phosphate groups to its substrate (van den Heuval and Harlow, 1993). Proteins from cellular lysates were immunoprecipitated with anti-Ha and Western probed for E2F4, in this way only transfected E2F4 was considered (Fig. 6.2A). In the presence of cdk2dn the upper, slowest migrating form that is indicative of an E2F4 phosphorylation event is absent (Fig. 6.2A, lane2). A slower migrating form that is found with cdk2wt (Fig. 6.1A lane 2), is apparent in the presence of p130 and to a lesser extent when E2F4 is expressed on its own (Fig. 6.2A, lane 3,4). These results suggest that cdk2 activity can phosphorylate E2F4 and, in the presence of p130, E2F4 phosphorylation is enhanced.

The potential of p130 to alter the cdk2 phosphorylation of E2F4 was tested next. Previous reports employing recombinant purified GST fusion proteins or baculovirus

infected lysates show an inhibition of kinase activity (Castano et al., 1998; Coats et al., 1999; Woo et al., 1997). However, others find that a p130 associated kinase can phosphorylate E2F4 (Vairo et al., 1995). This potential was verified by experiments that relied on endogenous cdk2 in C33A cells that were transfected with plasmids expressing E2F4-Ha and p130 (Fig. 6.2B). The cellular lysates normalized for protein content were divided into two parts. Each part was immunoprecipitated by a polyclonal cdk2 antibody and subjected to an *in vitro* kinase assay with ^{32}P-ATP (materials and methods). Any protein in the immune complex with cdk2 potentially could be phosphorylated. To determine whether Ha-tagged E2F4 was one of the phosphorylated proteins, the contents of the kinase assay, from one of the two parts, were re-immunoprecipitated by anti-Ha. The results revealed that E2F4-Ha was part of the immune complex with cdk2 as it was re-immunoprecipitated (Fig. 6.2B, lane3). In addition, more E2F4-Ha was phosphorylated in the presence of p130, as the amount of E2F4-Ha that was re-immunoprecipitated was increased (Fig. 6.2B compare lanes 3 and 5). These experiments implied that E2F4 is in a complex with cdk2 and that cdk2 is a p130 associated kinase that transfers phosphate to E2F4 *in vitro*.

The potential of p130 to alter the cdk2 phosphorylation of E2F4 by limiting the scope of the *in vitro* kinase reaction to an E2F4 specific immune complex was also assayed. In this approach, E2F4 and cdk2wt on their own and E2F4 together with cdk2wt, cdk2dn, or p130, were over expressed in C33A cells. Lysates of the transfected cells were immunoprecipitated for E2F4 via the Ha epitope and subjected to an *in vitro* kinase assay with ^{32}P-ATP (Fig. 6.2C). No phosphorylation of E2F4 is present with co-expression of cdk2dn and only slight phosphorylation when E2F4 is solely expressed (Fig. 6.2C lanes 1,5). However, the level of E2F4 phosphorylation is increased by co-expression with cdk2wt and further augmented by p130 (Fig. 6.2C, lanes 3,4). These results thus corroborate the previous findings suggesting that a cdk2 activity is involved in

the phosphorylation of E2F4. In addition, the *in vivo* and *in vitro* data indicate that E2F4 which cannot bind to cyclin A and cyclin E directly can nonetheless be phosphorylated by cdk2 with the aid of p130.

6.2.iii An E2F4 mutant deficient in pocket protein binding cannot be phosphorylated by cdk2

From these experiments, it is surmised that an E2F4 mutant that is unable to bind pocket proteins should not be phosphorylated by cdk2. This assumption was tested using a mutant of E2F4, designated 382CT (Fig. 3.10A and 6.3A). The Ha-tagged 382CT deletion mutant was tested for binding to p130 by Western blotting techniques. In this experiment, 382CT was transfected, solely or in combination with p130, into C33A cells (Fig. 6.3B). The transfected lysate was immunoprecipitated in parallel by anti-p130 and anti-Ha antibodies and probed by a polyclonal p130 antibody. Only the full length E2F4-Ha bound to p130 (Fig. 6.3B compare lanes 4 and 6).

The potential of 382CT to become differentially phosphorylated as judged by the full length was characterized next. This was accomplished by over expressing 382CT or E2F4-Ha as a positive control in C33A cells. The transfected cells were metabolically labeled by ^{35}S- methionine/cysteine and immunoprecipitated in duplicate by anti-Ha. To one of the immunoprecipitations of each group was added lambda phosphatase that is known to catalyze the removal of phosphate groups from threonines and serines. The result was the conversion of the slowest migrating forms of 382CT to a faster migrating one implying that phosphates were removed as was the case for wildtype E2F4 (Fig. 6.3C, lanes 3,4 and 1,2). The phosphorylation of 382CT was not as extensive as with the wildtype (Fig. 6.3C, compare lanes 2 and 4). This suggests the potential of 382CT to become phosphorylated in the absence of pocket protein binding, but not to the extent of the full length protein.

Figure 6.3 E2F4 deficient in pocket protein binding is not phosphorylated by cdk2 (A) Schematic representation of E2F4 in comparison with 382CT. Functional and structural regions are noted. (B) C33A cells were transfected with plasmids expressing 382CT (lane 1,2) or p130 in combination with E2F4-Ha (lane 3,4) and 382CT (lane 5,6). Cellular lysates, normalized for their protein content, were immunoprecipitated for p130 (ctp130) (lane 1,3,5) and Ha-tagged 382CT and E2F4 with 12CA5 (lane 2,4,6) 40 hr. after transfections. The immune complexes were electrophoresed on a 7.5% polyacrylamide gel, blotted and probed for p130 (C-20). (C) C33A cells were untransfected or transfected with plasmids expressing E2F4-Ha or 382CT. 40 hr. after transfection cells were metabolically labeled with ^{35}S-methionine/cysteine. Cellular lysates, normalized for their protein content, were divided into two and immunoprecipitated for Ha-tagged proteins with 12CA5. One of the immunoprecipitations of each transfected set was treated with lambda phosphatase. Treated and untreated immune complexes were electrophoresed on a 7.5% polyacrylamide gel and subjected to fluorography. (D) C33A cells were untransfected or transfected with plasmids expressing 382CT or 382CT together with cdk2wt. The cells were metabolically labeled with ^{35}S-methionine/cysteine 40 hr. after transfection. Cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged 382CT by 12CA5. Immune complexes were loaded onto a 7.5% polyacrylamide gel, electrophoresed and subjected to fluorography. (E) C33A cells were untransfected or transfected by plasmids expressing E2F4-Ha or 382CT. Cellular lysates, normalized for their protein content, were immunoprecipitated for cdk2 and subjected to an *in vitro* kinase assay with ^{32}P-ATP. SDS release buffer was added to the kinase complexes and the resulting soluble fractions re-immunoprecipitated for Ha-tagged proteins with 12CA5. Re-immunoprecipitated proteins were electrophoresed on a 7.5% polyacrylamide gel.

The potential ability of cdk2 to catalyze the observed phosphorylation of 382CT was assessed *in vivo* using transient transfections of C33A cells with 382CT in the presence or absence of cdk2. If cdk2 phosphorylates the 382CT mutant then a shift to a slower gel migrating form of 382CT as was discernible for E2F4 in Figure 6.1A would be expected. No shift of 382CT in the presence of cdk2 was apparent when metabolically radiolabeled transfected cells were immunoprecipitated by anti-Ha (Fig. 6.3D). This result suggested that the pocket binding region of E2F4 is necessary for phosphorylation by cdk2.

The ability of endogenous cdk2 to phosphorylate 382CT using an *in vitro* kinase reaction was assessed as in Figure 6.2B. Transfected cell lysate containing overexpressed 382CT was immunoprecipitated for cdk2 and subjected to an *in vitro* kinase reaction. The phosphorylated components of the kinase reaction were made available for re-immunoprecipitation by anti-Ha. If 382CT was phosphorylated by cdk2 then it would be re-immunoprecipitated. In this experiment, only the full length E2F4-Ha was re-immunoprecipitated despite the potential of 382CT to become phosphorylated (Fig. 6.3E compared to 6.3C). Therefore, taken together with the previous results, a mutant of E2F4 that does not bind to pocket proteins cannot be phosphorylated *in vivo* and *in vitro* by cdk2.

6.2.iv A p130 mutant that does not bind cyclin A and cyclin E cannot facilitate the E2F4 phosphorylation by cdk2

E2F4 was assessed to determine whether it could be phosphorylated in the presence of a p130 mutant that cannot bind to cyclins E and A. The p130 mutant Δ620-697 designated as (-sp)p130 has a deletion of amino acids 620-697 in the spacer domain (Lacy and Whyte, 1997) (Fig. 6.4A). The deletion removes the Arg-Arg-Leu (RRL) binding

motif present in the spacer region that is required for binding to cyclins A and E. A homologous sequence motif is present on p107 and other cell cycle regulators (Adams et al., 1996). This mutant was first tested for its potential to interact with E2F4 by overexpressing its CMV vector into ChaGO cells with E2F4-Ha and Western blotting for p130. The Western blot revealed the ability of (-sp)p130 to interact with E2F4 despite the loss of cyclin E and A binding (Fig. 6.4B).

(-sp)p130 was analyzed to determine whether it was phosphorylated *in vitro* by endogenous cdk2 by re-immunoprecipitating for p130. This was examined by overexpressing (-sp)p130, and full length p130 as a positive control, in C33A cells. Cell lysates were immunoprecipitated for cdk2 and subjected to an *in vitro* kinase assay with ^{32}P-ATP. SDS release buffer was added to the kinase complex and p130 was re-immunoprecipitated. Only endogenous p130 and the exogenous wildtype p130 were phosphorylated and thus re-immunoprecipitated (Fig. 6.4C). The mutant p130 protein was not phosphorylated as it was not re-immunoprecipitated in this experiment. Therefore, this mutant of p130 that does not bind to cyclin E or A does not harbour cdk2 kinase activity. (-sp)p130 was investigated whether it could affect phosphorylation of E2F4 *in vivo*. If p130 is involved in E2F4 phosphorylation by cdk2 then (-sp)p130 would not be able to effect an E2F4 electrophoretic shift in mobility. This hypothesis was tested by co-expressing E2F4-Ha with (-sp)p130 or with wt p130 as a positive control. As in Figure 6.1A the transfected cells were radiolabeled by ^{35}S-methionine/cysteine and immunoprecipitated for E2F4 by the 12CA5 antibody. With co-expression of p130, the E2F4-Ha protein possessed a slower migrating band that was absent when co-expressed with (-sp)p130 (Fig. 6.4D compare lanes 2 and 3).

The differences in mobility of the upper bands were due to differential phosphorylation as verified by treating with lambda phosphatase. The same transfections

Figure 6.4 A p130 mutant that does not bind cyclin A and E is deficient in facilitating the cdk2 phosphorylation of E2F4. (A) Schematic representation of p130 compared with (-sp)p130. The A/B pocket domains as well as the spacer region are denoted. (B) ChaGO cells were untransfected (lane 5,6) or transfected with plasmids expressing E2F4-Ha in combination with (-sp)p130 (lane 1,2) and p130 (lane 3,4). Cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged E2F4 (12CA5) and p130 (ctp130) 40 hr. after transfection. The immune complexes were loaded onto a 7.5% polyacrylamide gel, electrophoresed, blotted and probed for p130 (C-20). (C) C33A cells were transfected with plasmids expressing E2F4-Ha or E2F4-Ha in combination with p130 and (-sp)p130. Cellular lysates, normalized for their protein content, were Iped for cdk2 and subjected to an *in vitro* kinase assay with ^{32}P-ATP. SDS release buffer was added to the immune complexes and the released proteins re-immunoprecipitated for p130 (ctp130). The re-immunoprecipitated immune complexes were electrophoresed on a 7.5% polyacrylamide gel and visualized by autoradiography. (D) C33A cells were transfected with plasmids expressing E2F4-Ha in combination with p130 or (-sp)p130, 40 hr. after transfection cells were metabolically labeled with ^{35}S-methionine. Cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged E2F4 by 12CA5 loaded onto a 7.5% polyacrylamide gel, electrophoresed and subjected to fluorography. (E) The same transfections and metabolic labeling as in (D) were performed. Lysates from each transfection were divided into two and immunoprecipitated with anti-Ha. One immunoprecipitation from each transfection was treated with lambda phosphatase. (F) C33A cells were transfected with plasmids expressing the indicated proteins. 40 hr. post transfection, cellular lysates were immunoprecipitated for Ha-tagged E2F4 by 12CA5 and subjected to an *in vitro* kinase assay with ^{32}P-ATP. Loaded on to a 7.5% polyacrylamide gel, electrophoresed and visualized by autoradiography.

as in Figure 6.4D were employed and transfected radiolabeled cell lysates were immunoprecipitated in duplicate for Ha-tagged E2F4 by the 12CA5 antibody. To one of the immunoprecipitations from each set, lambda phosphatase was added. All the E2F4 forms present with p130 and (-sp)p130 represented differential phosphorylation as they collapse to one predominant band with the addition of phosphatase (Fig. 6.4E).

The *in vitro* potential of E2F4 to become phosphorylated in the presence of (-sp)p130 was also examined. If p130 facilitates phosphorylation then in the presence of a cyclin-binding-defective mutant E2F4 should not be phosphorylated by cdk2. This was investigated by transiently transfecting C33A cells with E2F4-Ha or E2F4-Ha in combination with p130, (-sp)p130 and cdk2. Cellular lysates were immunoprecipitated for Ha-tagged E2F4 and immune complexes subjected to an *in vitro* kinase assay with ^{32}P-ATP. (-sp)p130 co-expressed with E2F4-Ha resulted in an almost complete abolition of E2F4 phosphorylation as compared to when E2F4-Ha is expressed on its own (Fig. 6.4F compare lanes 2 and 5). As before, the addition of p130 increased the phosphorylation of E2F4 (Fig. 6.4F, compare lanes 2 and 6).

6.2.v E1a impedes the ability of p130 to facilitate the phosphorylation of E2F4 by cdk2

Another way to determine if the E2F4 interaction with p130 was necessary for cdk2 targeted phosphorylation made use of E1a. E1a binding to pRb disrupts its interaction with E2F (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991; Zamanian and La Thangue, 1992). Thus, it is surmised that p130 would be sequestered by E1a thereby eliminating its interaction with E2F4. The end result would be an inefficient cdk2 phosphorylation of E2F4. The potential for E1a to disrupt the p130 interaction with E2F4 was first appraised. This was accomplished by overexpressing

Figure 6.5 E1a impedes the p130 facilitated phosphorylation of E2F4. (A) C33A cells were untransfected (lane 1,2) or transfected with plasmids expressing E2F4-Ha with p130 (lane 3,4) or (-sp)p130 (lane 5,6) or E2F4-Ha and E1a together with p130 (lane 7,8) or (-sp)p130 (lane 9,10). 40 hr. post transfection cellular lysates, normalized for their protein content, were immunoprecipitated for Ha-tagged E2F4 with 12CA5 or p130 (ctp130) as indicated. Immune complexes were loaded onto a 7.5% polyacrylamide gel, electrophoresed and blotted. The blot was probed for p130 (C-20). (B) C33A cells were untransfected or transfected with plasmids expressing the indicated proteins (above figure). Cellular lysates, normalized for their protein content, were immunoprecipitated for cdk2 and subjected to an *in vitro* kinase assay and re-immunoprecipitated for Ha-tagged E2F4 as in Figure 6.3E.

these proteins in ChaGO cells. Cellular lysates were immunoprecipitated in parallel for Ha-tagged E2F4 and p130. The immunoprecipitations were examined for the presence of p130 by the Western blotting technique. The results of this experiment revealed that p130 no longer interacted with E2F4 in the presence of E1a (Fig. 6.5A, compare lanes 3 and 7). Next the potential for E2F4 to become phosphorylated in the presence of E1a was tested. In this case ChaGO cells were transfected by p130 and E2F4-Ha in the presence of E1a. Cellular lysates were immunoprecipitated for endogenous cdk2, subjected to an *in vitro* kinase assay with ^{32}P-ATP and re-immunoprecipitated for Ha-tagged E2F4. With the co-expression of E1a no re-immunoprecipitation of E2F4 was obtained (Fig. 6.5B). Therefore, E1a depletes the potential for E2F4 to become phosphorylated by cdk2 as it sequesters the bridging molecule p130.

6.2.vi cdk2 is not sufficient to abrogate the p130 suppression of E2F4 mediated transactivation

p107 and p130 can suppress E2F4 mediated transactivation (Beijersbergen et al., 1994; Ginsberg et al., 1994; Johnson, 1995; Vairo et al., 1995). Cdk4 activity can rescue p107 suppression of E2F4 mediated transactivation, but cdk2 cannot (Beijersbergen et al., 1995). To date, the cdk effect on the p130 suppression of E2F4 transactivation potential has not been reported. In this regard, the functional consequence of cdk activity on the p130-E2F complex was assessed with transient transfection assays using an E2F-CAT reporter. In these experiments, C33A cells were transfected with the E2F-CAT reporter, E2F4 and Dp-1 on their own or co-expressed with p130 (Fig. 6.6). The potential effect of the cdks was determined by co-expressing p130 with cyclin D1 and cdk4 or cyclin E and

cdk2. In the presence of p130 the CAT activity is reduced to about 25%. As with p107, the co-expression of cyclin D1/cdk4 with p130 rescued the suppression of CAT activity. The result was not to completely restore the activity levels. This is not possible because E2F4 is also regulated by cdk4 and the end result of this regulation is a reduction of E2F4 binding potential (Chapter 3). Cyclin E/cdk2 had no effect on the p130 regulated CAT activity. These results imply that cdk2 and cdk4 activities on the p130 control of E2F4 mediated transactivation is the same as for p107. The role of p130 facilitated phosphorylation of E2F4 by cdk2 is not apparent, but it may play some other role such as influencing higher order complexes.

Figure 6.6 Cdk2 activity is not sufficient to disrupt the p130 suppression of E2F4 mediated transactivation. C33A cells were co-transfected with 2ug of $E2F_4$-CAT reporter plasmid, 2ug of pGL3-Control as an internal control with and without 2ug of pCMVHAE2F-4 and 2ug of pcmvDp-1 and 5ug of expression vectors carrying the proteins for cdk4wt, cyclin D1, cyclin E, p130 and cdk2wt as indicated. Each transfection may also contain pcDNA3 to make up to a total of 23 ug of transfected DNA. CAT activities were determined for the various transfections and the values were normalized for the corresponding luciferase activities. The results are calculated relative to the E2F4/Dp-1 activity which is set at 100. The results are representative of at least three different experiments with error bars representing standard error of the mean.

6.3 Conclusion

The results presented verify that the phosphorylation of E2F4 by cdk2 is possible *in vitro* and *in vivo*. Furthermore, the findings indicate that p130 can function as a facilitator to the E2F4 phosphorylation by cdk2. The binding studies presented describe the inability of cyclin A to interact with E2F4 as well as cyclin E. As E2F4 can be phosphorylated by the kinases associated with p107 and p130 and cdk2 *in vitro* and *in vivo*, it suggests that a component should be able to bind to cyclins A and E and/or cdk2 in complex with E2F4. Candidates for this are p107 and p130 that can form stable complexes with E2F4, cyclin A or E and cdk2 (Cobrinik et al., 1993; Lees et al., 1992; Mudryj et al., 1991; Shiyanov et al., 1996).

Data for p107 with reconstituted recombinant proteins suggest that it might also be involved in bridging the kinase activity to E2F4. Purified E2F4 serves as a substrate for both cyclin A and E/cdk2 catalytic activity only in the presence of an N-terminal truncated mutant of p107 that lacks the cdk2 inhibition motif (Woo et al., 1997). The ability of p130 to facilitate the phosphorylation of E2F4 is supported by three different approaches. The *in vivo* over expression studies demonstrate the increase in phosphorylation for E2F4, by mobility shift, with p130 co-expression. The *in vitro* kinase assays for immune complexes containing transfected E2F4 revealed that the co-expression of p130 potentiated the phosphorylation of E2F4. In addition, a mutation of p130 that does not bind cyclin A or E is unable to phosphorylate E2F4. In a second approach the question of facilitated cdk2 phosphorylation is assessed by employing a mutant of E2F4 that is incapable of binding pocket proteins. Although this mutant is differentially phosphorylated it nonetheless lacks the potential to be a substrate for cdk2 in the *in vitro* kinase assays. A third approach makes use of E1a proteins that bind to p107 and p130 nullifying their E2F4 binding potential. In this context E1a is able to reduce the efficiency of cdk2 mediated E2F4 phosphorylation as well. These results hint at a functional basis for the E1a elimination of

the E2F4 interaction with p130; that is, the abrogation of the p130 kinase bridging function. A role for p130 facilitated phosphorylation of E2F4 by cdk2 is not apparent from transient transfection assays. This suggests that phosphorylation of E2F4 in complex with p130 might influence higher ordered structures.

Chapter 7. Conclusion and Discussion

7.1 Cyclin D1/cdk4 directly regulates E2F4

Previous reports suggest that the sole functional outcome of cyclin D kinase is the phosphorylation of the Rb family. D-type cyclins with their kinase partners cdk4 or cdk6 phosphorylate pRb in the mid to late G1 phase of the cell cycle promoting the release of E2F. The Rb-free E2F is able to activate the genes required for S-phase progression (Ewen et al., 1993; Horton et al., 1995; Matsushime et al., 1992). There is ample evidence that this is an obligatory pathway. Experiments demonstrate that a cell cycle block in G1 from the ectopic addition of the specific cdk4/cdk6 inhibitor p16 (Guan et al., 1994; Hirai et al., 1995; Lukas et al., 1995; Medema et al., 1995) or the targeted disruption of the cyclin D1 protein (Baldin et al., 1993; Lukas et al., 1995; Quelle et al., 1993) requires a functional pRb protein. Recently, p130 and p107 have also been implicated as targets of D-type cyclin activity and their phosphorylation by cdk4 leads to a loss of E2F binding, culminating in the derepression of gene expression (Beijersbergen et al., 1995; Dong et al., 1998; Johnson, 1995; Xiao et al., 1996). The functional outcome of kinase modification for other potential targets of cyclin D/cdk4 are inconclusive (Hirai and Sherr, 1996; Inoue and Sherr, 1998).

Experimental results presented in this thesis, support the view that cyclin D1/cdk4 directly regulates E2F4 by the disruption of E2F4 DNA binding potential. This is analogous to the cyclin A/cdk2 activity which is necessary for the attenuation of E2F1 that occurs at S-phase (Krek et al., 1994; Krek et al., 1995; Xu et al., 1994). Cyclin A control of E2F1 requires that it bind directly to E2F1 at a region that is also present in E2F2 and E2F3, but is not present in E2F4 and E2F5. The recruitment of cdk2 by cyclin A causes the phosphorylation of E2F-1 and/or DP-1 interfering with their ability to bind to DNA.

The *in vivo* binding data in Chapter 3 demonstrate E2F4, E2F5 and Dp-1 bind efficiently to cyclin D1. E2F1, E2F2, and E2F3 are less efficient in binding to cyclin D1. Diminished binding for E2F1, E2F2 and E2F3 is not related to a reduced expression as all the E2Fs and Dp-1 are expressed to comparable concentrations. Reduced binding might be a reflection of the limited availability of endogenous interacting proteins or an indirect interaction. One possibility is that the cyclin D1 interaction with E2F1, E2F2 and E2F3 is bridged by Dp-1. It is notable that cyclin D1 interacts strongly with E2F4, E2F5 and Dp-1 representing the components involved in the E2F binding activity at quiescence (Beijersbergen et al., 1994; Ginsberg et al., 1994; Ikeda et al., 1996; Lindeman et al., 1997; Moberg et al., 1996; Muller et al., 1997; Smith et al., 1996; Verona et al., 1997). Cyclin D1 is the first G1 cyclin to be expressed from quiescence representing the regulatory subunit for early activated cdk in mid G1 suggesting a functional role for cyclin D1 in the regulation of E2F4 (Matsushime et al., 1992; Resnitzky and Reed, 1995). This correlates to a time when certain promoter sites are no longer bound by E2F4.

Results using different experimental approaches demonstrate that cyclin D1/cdk4 activity is sufficient to disrupt the E2F4 DNA binding potential. Co-expression of D-type cyclins and cdk4 decreases E2F4 mediated transactivation of an E2F promoter driven gene in a pocket protein independent manner. EMSA provided insight into the nuclear and cytoplasmic E2F4/Dp-1 DNA binding potential with co-expression of cyclin D1/cdk4 in a pocket protein independent manner. E2F4 and Dp-1 were present in nuclear as well as cytoplasmic fractions despite the addition of cdk4 and cyclin D1. Others have found that E2F4 is influenced by factors that affect its stability and cellular compartmentalization (Hateboer et al., 1996; Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). However, the loss of DNA binding in the presence of active cdk4 is not due to changes in E2F4 protein stability or to redistribution of E2F4 or Dp-1 from the nuclear to the cytoplasmic compartment.

The loss of DNA binding might correlate to the disruption of an E2F4 interaction with Dp-1. Evidence that activated cdk4 has disrupted the interaction are from *in vivo* phosphopeptide mapping experiments. Phosphopeptide maps revealed that some phosphorylated forms of E2F4 cannot bind to Dp-1. The possibility for Dp-1 phosphorylation contributing to the effect cannot be ruled out. The phosphorylated forms of E2F4 appear identical in both cyclin D1/cdk4 positive and negative nuclear extracts suggesting that the E2F4/Dp-1 interaction is unchanged. This raises the possibility that some E2F phosphorylated forms are unable to bind DNA yet continue to bind Dp-1. Alternative explanations are the presence of an imperceptible phosphorylation event on E2F4 or a Dp-1 modification. The latter explanation has some validity as cyclin D1 binds directly to Dp-1 and might target it for phosphorylation by cdk4 leading to the interruption of binding with its dimerization partner. This has been demonstrated for the E2F1/Dp-1 DNA interaction in the presence of cyclin A/cdk2 (Xu et al., 1994).

The domain mapping studies show two sites on E2F4 that can bind to cyclin D1. These are located near the C-terminal pocket protein binding region and the N-terminal Dp-1 dimerization domain. The removal of the C-terminal binding site is sufficient to displace the ability of cyclin D1 activated kinase to regulate E2F4/Dp-1 DNA binding potential. After many attempts, the E2F4 interaction for pocket proteins or Dp-1 has not been competed for by cyclin D1. A possibility that this might occur is provided by the findings of the cyclin D1 effect on the V-myb and the myb like DMP1 transcription factors (Ganter et al., 1998; Inoue and Sherr, 1998). D-type cyclins specifically repress transcriptional activity for these molecules by direct interaction with their DNA binding domains in a cdk4 independent manner.

7.2 A potential early G1 timing for the cyclin D1/cdk4 regulation of E2F4

The cdk4 activity directed toward E2F4 occurs presumably during early events of the cell cycle and may be required to derepress or activate genes from promoters that are bound by E2F4 at G0. The expression pattern for E2F4, unlike E2F1, E2F2, and E2F3, is not regulated in a cell cycle dependent manner (Hsiao et al., 1994; Johnson et al., 1994; Leone et al., 1998; Moberg et al., 1996; Sears et al., 1997; Vairo et al., 1995). E2F4 is the predominant E2F family member in G0 and, with Dp, it forms a complex with p130 (Beijersbergen et al., 1994; Cobrinik et al., 1993; Ginsberg et al., 1994; Lindeman et al., 1997; Moberg et al., 1996; Muller et al., 1997; Smith et al., 1996; Verona et al., 1997). At this point, the E2F4 binding potential under the influence of cyclin D1/cdk4 can invoke distinct outcomes that are promoter dependent. As stated previously, a large body of evidence indicates that E2F binding sites are not identical. They are repressed or activated differently by different E2Fs influenced by their pocket protein partners and other interacting transcription factors (DeGregori et al., 1997; Fry et al., 1997; Hurford et al., 1997; Lin et al., 1996; Lindeman et al., 1998; Tao et al., 1997; Tommasi and Pfeifer, 1997; Yamasaki et al., 1996).

Two models (that may not necessarily be mutually exclusive) are proposed to explain how the action of cyclin D1/cdk4 affects E2F4-p130 bound promoters (Fig. 7.1). In one scenario, p130 might be phosphorylated by a kinase other than cdk4 early in G1, before the induction of the D-type cyclins. The result of p130 phosphorylation would be to disrupt its complex formation with E2F4. With the elimination of p130 binding a small window of gene expression potentiated by E2F4 might ensue. For example others have shown that E2F4 is required for the transactivation of cyclin D1 gene expression (Watanabe et al., 1998). Transient transfection assays presented in this thesis and elsewhere indicate that E2F4 is able to mediate transactivation of simple E2F promoter

Figure 7.1 Hypothesis of cell cycle timing for E2F4 control by cyclin D/cdk4. E2F4, bound to Dp-1 and p130, represents the E2F activity in G0. This is a point in the cell cycle when promoter sites that bind E2F are repressed. Early in G1, p130 is removed by phosphorylation, possibly by cyclin D/cdk4. For some promoters gene transcription ensues. With the removal of p130, E2F4 DNA binding potential is lost early in G1 by Cyclin D1/cdk4 targeted phosphorylation of E2F4 and possibly Dp-1. The result is that the E2F4/Dp-1 active promoters are silenced and others are derepressed. At a later time in S phase the targeting of E2F1 and Dp-1 for phosphorylation removes the E2F1/Dp1 binding potential thereby silencing these promoters.

constructs (Beijersbergen et al., 1994; Ginsberg et al., 1994). Later in G1, as D-type cyclins are expressed, cdk4 could act on E2F4 disrupting its DNA binding potential. This possibility is supported by the p130 potential phosphorylation in a cdk independent manner (Mayol et al., 1996). The other scenario has p130 and E2F4 both phosphorylated by cdk4 at the same time. The result would be the breakup of the E2F4-p130 complex and the disruption of the E2F4 DNA binding ability. In both scenarios the major E2F activity, E2F4, could be replaced by E2F1, E2F2 or E2F3 in mid G1. The newly acquired E2F activity would then be silenced at S-phase by cyclin A/cdk2.

The loss of E2F4 DNA binding potential by cyclin D1/cdk4 correlates with the loss of E2F4 binding in G1, that is required for gene expression for many E2F element containing promoters (Hiyama et al., 1998; Iavarone and Massague, 1999; Johnson, 1995; Johnson et al., 1994; Ohtani et al., 1996; Ohtani et al., 1995; Sears et al., 1997; Tommasi and Pfeifer, 1995; Watanabe et al., 1998; Zwicker et al., 1996). This corresponds to the known timing for the subcellular re-distribution of E2F4 to the cytoplasm that is thought to occur at mid G1 and the timing of the induction of cyclin D1/cdk4 kinase activity (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997).

7.3 Consequences at E2F4 bound promoter sites by cyclinD1/cdk4 activity

As not all E2F4 promoter binding sites are equal, there are three possible outcomes resulting from the cyclin D1/cdk4 direct regulation of E2F4. One outcome is the elimination of E2F4 at its promoter element without replacement by another E2F family member. Evidence for this scenario is from *in vivo* footprinting experiments examining the E2F promoter site of the B-myb and cdc2 genes (Tommasi and Pfeifer, 1995; Zwicker et al., 1996). The B-myb and cdc2 promoters are occupied by E2F4 at G0 and early in G1 but remain unoccupied at later stages of the cell cycle allowing the genes to be transcribed. A small window of E2F4 enhanced activation might account for the observed gene

expression, but the neutralization of E2F4 DNA binding at these promoters in G1 is consistent with the timing of cyclin D1/cdk4 activity. Other evidence is from studies on the human E2F1 promoter that show the E2F promoter site is derepressed by cyclin D1/cdk4 activity. Derepression involves the removal of a G0 E2F that binds p130, most likely E2F4 (Johnson, 1995; Johnson et al., 1994; Smith et al., 1996). A mutation of the binding site relieves negative control of the E2F1 promoter in early G1 and a mutant E2F that is incapable of transactivation competes out the bound E2F leading to gene activation. This implies that derepression can only occur if the DNA site is no longer bound.

A second possible outcome of activated cyclin D1/cdk4 is the substitution of E2F4 by other E2Fs during early G1. Evidence that this occurs is from reports that indicate a temporal control of the same promoter site by different E2Fs (Hiyama et al., 1998; Karlseder et al., 1996; Ohtani et al., 1996; Ohtani et al., 1995; Sears et al., 1997; Watanabe et al., 1998). For example, a region of the human cyclin D1 promoter is activated by E2F4 early in G1 whereas the same site is required for repression by E2F1 at a later time (Watanabe et al., 1998). Another example, is for an E2F binding site in the murine thymidine kinase promoter for which *in vivo* footprinting describes the presence of constitutively bound E2F activity (Karlseder et al., 1996). As the predominant member at this stage of the cell cycle, E2F4 is bound to this promoter when gene expression is shut down during quiescence. However, activation of Tk gene expression from this promoter site requires Sp1 to act in synergy with E2F1 not E2F4. These experiments suggest that E2F4 must be replaced by E2F1 for activation to occur.

Finally, the DHFR promoter represents a third class of E2F4 bound promoter sites. The DHFR promoter, homologous in human, mouse and hamster, has E2F4 bound to it throughout the cell cycle, despite cyclin D1/cdk4 activity (Leone et al., 1998; Wells et al., 1996; Wells et al., 1997). The promoter site contains a dyad E2F binding site able to bind to two E2F molecules simultaneously. This unusual conserved element might protect E2F4

from phosphorylation or increase its DNA binding stability when E2Fs are doubly bound (Wells et al., 1996). In addition, this type of E2F4 binding site might be influenced by factors such as the promoter distance to the transcriptional start site, the presence of a TATA box and the influence of other transcription factors (Fry et al., 1997; Lin et al., 1996; Tommasi and Pfeifer, 1997).

7.4 Differential roles for cdk2 and cdk4

The phosphorylation of E2F4 by cdk2, mediated by p130, provides a starting point to explain the stable complex formation of p130 or p107 with cyclins A or E and cdk2 (Fig. 7.2). The initial link between cdk2 activity and E2F4 stems from data reporting the differential phosphorylation of E2F4 in a cell cycle dependent manner (Vairo et al., 1995). Furthermore, the ability of kinases associated with p107 and p130 to phosphorylate E2F4 *in vitro* suggested that cdk2 might be involved (Ginsberg et al., 1994; Vairo et al., 1995). The *in vivo* data, presented in the thesis, details a shift to a slower gel migrating form of E2F4 when the cells were transiently transfected with cdk2, but not when co-expressed with cdk2dn. These results correlate with the ability of wildtype cdk2, but not cdk2dn, to phosphorylate E2F4 *in vitro* in the co-expression studies.

In this thesis EMSA and CAT assays suggested that cdk2 phosphorylation of E2F4 and possibly p130 are not sufficient to breakup the DNA binding or pocket protein binding ability of E2F4. This implies that cdk2 cannot rescue suppression of E2F4-mediated repression or transactivation, a finding that has been noted for p107 (Beijersbergen et al., 1995). In contrast, the results herein demonstrate that cyclin D/cdk4 activity can rescue suppression by p130 of E2F4 mediated activation by disrupting the p130-E2F4 interaction. This finding, together with published data, suggest that cyclin D activated kinase has the potential to disrupt the pocket protein interaction with E2F4 (Fig. 7.2). Others have reported the cdk2 potential to abrogate the interaction between E2F4 and p107 in EMSA

Figure 7.2 cdk4 vs cdk2 regulation of the p130-E2F4 complex. Cyclin D/cdk4 activity, by targeted phosphorylation of the pocket protein, causes the disruption of the pocket protein interaction with E2F4. Activated cdk2 phosphorylates E2F4 in the presence of p130 (and possibly p107) with an unknown outcome.

(Zhu et al., 1995). They demonstrate the elimination of the pocket protein interaction with E2F4 in DNA binding studies in the presence of activated cdk2. The differences in their findings with the data presented in the thesis, might be due to the reliance on purified fusion proteins in their experiments or the possibility that p107 might function differently than p130.

At first glance the results presented in Chapter 6 appear to contradict the potential of p130 and p107 to inhibit cdk2 kinase activity, thereby implying that facilitated E2F4 phosphorylation would be impossible (Castano et al., 1998; Coats et al., 1999; Woo et al., 1997; Zhu et al., 1995). In p27-/- knockouts p130 binds to cyclin E in mitogen starved embryonic stem cells (MEFs) a function normally performed by p27 (Coats et al., 1999). However, inhibition is not a universal function as it does not occur in p27-/- hematopoietic stem cells and once mitogens are added this role for p130 is circumvented. Control of cyclin E/cdk2 activity by p130 may be tissue specific and cell cycle dependent. Other evidence suggests that cdk2 activity harboured by the pocket proteins is not always inhibited. Early reports show p130, p107 and E2F4 are phosphorylated in a cell cycle dependent manner (Mayol et al., 1995; Mayol et al., 1996; Xiao et al., 1996). Furthermore, p130 can be found in the presence of activated cyclin E and cyclin A (Hauser et al., 1997; Prall et al., 1998).

The hypothesis that E2F4 phosphorylation is facilitated by p130 during the cell cycle is strengthened by the findings that p130 can be found with cyclin E and cdk2 in asynchronous cells and E2F4 is bound at certain promoter sites throughout the cell cycle (Leone et al., 1998; Shiyanov et al., 1996; Wells et al., 1996; Wells et al., 1997). This provides an opportunity for E2F4 to be phosphorylated by cdk2, mediated by p130, at any point during the cell cycle. Perhaps cdk2 activity could affect higher ordered E2F4 structures on the DNA. For example, p130 is known to complex with RBP1 and E2F4 during growth arrest and differentiation (Corbeil and Branton, 1997; Lai et al., 1999).

E2F4 dependent transcription is suppressed by RBP1. Cdk2 phosphorylation of p130 and/or E2F4 might be necessary to disrupt their complex with RBP1 and allow gene expression from certain promoter sites. In this way cdk2 might function to modify the p130-E2F4 complex on certain promoter sites.

7.5 The p130 interacting domains for cyclin D1 and E2F4, E2F5 and Dp-1

The E2F binding sites for the pocket proteins are not identical. pRb and p107 require the A/B pocket for interaction whereas p130 requires a portion of the B domain. For p130 (and most likely p107) when E2F4 is interacting, the spacer element is necessary to complex with cyclins A and E and cdk2 providing a functional role. Cyclins A and E by binding to the spacer element are able to target the phosphorylation of the bound E2F4. pRb does not form a stable complex with the cyclins and might be unable to mediate this function. Differential control of E2F4 by the pocket proteins might be important in unraveling the rationale for E2F4 phosphorylation. In this way, aspects of E2F4 function might be manipulated with the aim of controlling cellular function.

The cyclin D1 interacting domain on p130 located at the C-terminus is dissimilar from the cyclin A and cyclin E interacting regions. This suggests that cyclin A and cyclin E target p130 for phosphorylation at sites that are distinct from cyclin D1. This possibility is supported by studies showing differential site phosphorylation by cyclin targeting of pRb. This implies that there might be different outcomes for phosphorylation by the different kinase activities, an idea that is strengthened by the functional outcomes of cdk2 versus cdk4 activity presented in this thesis.

The LxCxE domain present in the cyclin D family members previously was suggested to be required for complex formation with the pocket proteins (Dowdy et al., 1993). The finding that a mutant in the LxCxE motif of cyclin D1, Leu-x-Gly-x-Glu, can interact with the pocket proteins disputes this long held believe. The LxCxE motif is used

by DNA tumour virus oncoproteins to bind the pocket proteins (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). The LxCxE motif interaction with pRb has recently been determined. This was accomplished with crystallographic techniques for the 3-dimensional structure of a peptide of the E7 protein of papallomavirus bound to the A/B domain of pRb missing the spacer sequences (Lee et al., 1998). The peptide interacts specifically within the B domain, but the A domain is required for the interaction to occur as a stabilizing influence for the B domain. A hint that this mechanism of binding does not occur for the cyclin D family and corroboration of our findings are from reports indicating that C-terminal sequences of pRb are required for pRb to interact with cyclin D2 and D3 *in vitro* and the poor binding of the A/B pocket with cyclin D1 (Ewen et al., 1993). Other studies demonstrated that LxCxE mutants of cyclin D1 retain the ability to phosphorylate pRb *in vivo* and *in vitro* (Connell-Crowley et al., 1997; Horton et al., 1995). Alternatively, D-type cyclins might interact with two different binding motifs one requiring the LxCxE region. For example, different binding sites for cyclins A or E on p130 and p107 have been reported (Lacy and Whyte, 1997; Woo et al., 1997; Zhu et al., 1995). Thus in COS1 cells, cyclin D1 might only interact via LxCxE with other sites on pRb potentially modified, possibly by the binding of other factors or phosphorylation.

Chapter 8. References

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