Cell Cycle Control by Components of Cell Anchorage

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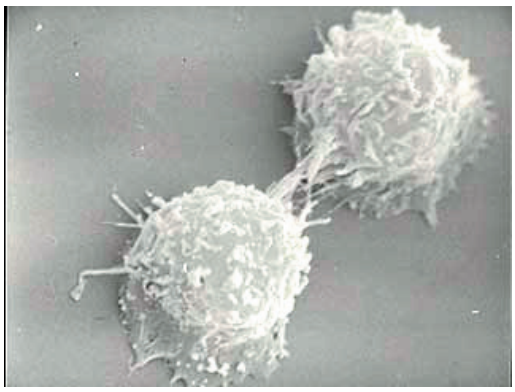

A Scanning electron microscopy image of a mammalian cell undergoing cell division. This picture is dowloaded and reprinted from the electron microscopy image gallery, Indigo Instrument's online catalogue, www.indigo.com, with permission from the copywrite owner.

Abstract

Extracellular factors, such as growth factors and cell anchorage to the extracellular matrix, control when and where cells may proliferate. This control is abolished when a normal cell transforms into a tumour cell. The control of cell proliferation by cell anchorage was elusive and less well studied than the control by growth factors. Therefore, we aimed to clarify at what points in the cell cycle and through which molecular mechanisms cell anchorage controls cell cycle progression. In addition, we wanted to clarify if two components involved in cell anchorage, the hyaluronic acid (HA) -binding domain of CD44 and p21-activated kinase 1 (PAK1), can control cell cycle progression.

Growth factors and cell anchorage have been considered to regulate cell proliferation exclusively by a joint control in the early/mid-G1-phase of the cell cycle. However, we found that in addition to this joint control, cell anchorage also controls progression through the late G1-phase as well as through the final cell division, cytokinesis. The control by cell anchorage in late G1-phase was found to be distinct from the control by growth factors in that it occurs after and independent of the normal control by serum, cyclin D-associated kinase activity, pRb and p107. In addition, we observed that although cells lacking anchorage could initiate the ingression of the cleavage furrow during cytokinesis, they could not complete cell division without cell anchorage. This anchorage-dependent control of cytokinesis could be mediated by various integrins as well as by integrin-independent cell anchorage.

Furthermore, we showed that the kinase-inhibitory domain of PAK1 could inhibit the induction of cyclin D1 and cyclin D2 as well as G1-phase progression. Surprisingly, the cell cycle inhibition of the PAK1 kinase-inhibitory domain appeared not to act through inhibiting PAK1 kinase activity, but through a different mechanism.

We also found that the recombinant hyaluronic acid-binding domain of the cell surface receptor CD44 (CD44-HABD) could inhibit endothelial cell cycle progression, angiogenesis and tumour growth. A mutant CD44-HABD without the ability to disturb the binding of CD44 to hyaluronic acid also inhibited cell cycle progression. Therefore, we rule out the possibility that the observed cell cycle inhibition was due to loss of CD44 binding to hyaluronic acid. We hypothesize that the recombinant domain of CD44 inhibits cell cycle progression by binding to an unidentified cell surface ligand.

A normal tissue cell can transform into a tumour cell only if it manages to overcome the control exerted by the surrounding extracellular matrix. Our results reveal that cell anchorage controls the cell cycle at additional steps as compared to growth factors. This finding underscores the importance of the cell anchorage-dependent control in the protection against cellular transformation and tumourigenesis. The anchorage of a cell to the surrounding extracellular matrix also needs to be altered at later stages of tumour progression. Thereby, further elucidation of how cell anchorage controls cell proliferation may provide means allowing the development of new therapies for cancer. This is further emphasised by our findings that domains of proteins involved in cell anchorage, such as CD44 and PAK1, can exert control of the cell cycle, and inhibit tumour cell growth.

Table of contents

List of papers

This thesis is based on the following papers, which are enclosed at the end of this thesis.

I **Gad A,** Thullberg M, Dannenberg JH, te Riele H, Strömblad S.
Retinoblastoma susceptibility gene product (pRb) and p107 functionally separate the requirements for serum and anchorage in the cell cycle G1-phase.
J Biol Chem. 2004 279:13640-13644.

II **Gad A***, Thullberg M*, Strömblad S.
Cell anchorage controls the final contraction of the acto-myosin ring during cytokinesis.
(Manuscript)

III Thullberg M, **Gad A**, Chernoff J, Strömblad S.
The kinase-inhibitory domain of p21-activated kinase 1 inhibits cell cycle progression independent of PAK1 kinase activity.
(Manuscript)

IV Päll T, **Gad A**, Kasak L, Drews M, Strömblad S, Kogerman P.
Recombinant CD44-HABD is a novel and potent direct angiogenesis inhibitor enforcing endothelial cell-specific growth inhibition independently of hyaluronic acid binding.
Oncogene 2004 23:7874-7881.

* these authors contributed equally to this work.

Abbreviations

APC	Anaphase promoting complex, ubiquitin ligase
CAK	Cdk activating kinase
Cdk	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
ECM	extracellular matrix
ERK	Extracellular signal –regulated kinase
FACS	fluorescence activated cell sorter
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GAP	GTP-ase activating protein
G1-phase	the gap-1 phase
G2-phase	the gap-2 phase
HA	Hyaluronic acid
H-Ras	Harvey-Ras
hTERT	the catalytic subunit of human telomerase
INK	inhibitor of Cdk4 and Cdk6
KID	p21-activated kinase inhibitory domain
MAb	monoclonal antibody
MAPK	Mitogen-activated kinase
MEF	mouse embryonic fibroblasts
MLCK	myosin light chain kinase
MMP	Matrix metalloproteinase
M-phase	the mitosis-phase
NHDF	normal human dermal fibroblasts
PAK1	p21-activated kinase 1
Plk	Polo-like kinase
pRb	Retinoblastoma susceptibility gene product
ROCK	Rho kinase 1 and 2
R-MLC	regulatory myosin light chain
S-phase	the DNA synthesis phase
SCF	Skp1/Cullin/F-box-complex, ubiquitin ligase
SV40T	the large T antigen of simean virus 40

Preface

All multicellular organisms depend upon a fine balance between cell proliferation, cell differentiation and cell death. In animals, this is crucial during embryonic development and to maintain tissue homeostasis. Strict control mechanisms that regulate cell proliferation have therefore evolved. These controls ensure that only the right cell, at the right time and in the correct extracellular enviroment, is allowed to proliferate. The extracellular factors that control when, and where, cell may proliferate, include growth factors and cell anchorage to the extracellular matrix (ECM). All cells constantly achieve genetic alterations in their genomes, which may change their characteristics. Occasionally, such changes result in a new organism with an increased fitness and a survival advantage and constitute thereby the basis for evolution. But, mostly such changes are detrimental for the balance between cells, and thus, for the organism. The organism has therefore developed controls of genetic integrity that correct genetic changes, or if it fails to do so kills the cell. Despite this, genetic changes occur, and sometimes these changes allow the cell to escape the extracellular controls of proliferation. The loss of these controls result in a cell that is less dependent on extracellular factors, can divide in an uncontrolled manner and therefore may cause tumour growth in animals. The anchorage-dependent control of cell proliferation must be disturbed in order to transform a normal cell into a tumour cell and this control is also altered at later stages in tumour pathology. The formation of new blood vessels, angiogenesis, that infiltrates the tumour provides the cells with nutrients and is required to establish a primary tumour. Angiogenesis may take place only if the normal control of cell anchorage on endothelial cells is disturbed. In addition, an altered control by cell anchorage on cell proliferation is required also for metastasis.

Studies of the cell anchorage-dependent control of normal cell proliferation as well as the defects in this control of tumour cells, will enable us to understand how tumour cells inactivate this important control of cell proliferation. This is of importance, since it can provide information that help in the development of potential cures for cancer.

Introduction

The Cell Cycle Model

Cell cycle

A model called the Cell cycle is commonly used to describe one round of cell division. According to this model, a cell division cycle is divided into the following four phases: The DNA synthesis phase (S-phase) in which the genome is replicated. The M-phase (M for mitosis) where the chromsomes are segregated and cells prepare for the final cell division. The Gap1- (G1) and Gap 2- (G2) phases are intermediate phases that separate M-phase and S-phase. Cells that start a new round of cell division enters the cell cycle G1-phase. This is regardless of if the cell just left a previous round of the cell cycle or if it starts proliferating from a resting, quiescent, stage. During this period, cells are receptive for either inhibitory or stimulating extracellular signals (79). The extracellular factors that control cell proliferation include soluble growth factors and cell anchorage to an appropriate extracellular matrix. The contact between cells is also of importance, since cell proliferation is inhibited by cell-cell contact (35). Cells has long been regarded to be susceptible to extracellular signals until a point in the mid G1-phase named the Restriction point (16, 141). After this point, cells have been considered irreversibly committed to go through the rest of the cell cycle and divide independently of extracellular signals. Thus, a molecular switch has been suggested to take place at the time of the Restriction point, which makes cells independent of extracellular factors (16, 79, 141, 108). The cell grows substantially in size during the G1-phase and protein synthesis is required for progression through the G1-phase (97). Cell cycle progression only occurs if the cell has reached a correct size, and coordination of cell growth and cell cycle progression is carried out under this period (58). If not all conditions are met the cell either stop in the G1-phase or rest in a quiescent stage, the G0-phase. Most of the cells in the body reside in a resting stage. This arrest can be temporal but also irreversible, which is the case in senescence and terminal differentiation. However, upon proper stimulation, the cell passes the Restriction point and starts protein synthesis of S-phase inducing genes. These genes allow the cell to start, continue and complete DNA replication during S-phase. The G2-phase is a period where the cell controls the quality of the replicated DNA and also accumulates components needed for mitosis (120). The G1-, S- and G2-phases are sometimes called Interphase, referring to the time between two mitoses. M-phase starts when the chromosomes condense. Mitosis is followed by ingression of the cleavage furrow that parts the two daugher cells in the final cell division, cytokinesis.

Molecular basis of cell cycle regulation

A specific family of serine/threonin kinases called cyclin-dependent kinases (Cdk) drive the cell cycle by the sequential phosphorylation of different targets (120). Since appropriate cell proliferation is crucial for the organism, the regulation of these kinase activities is rigorous. This control ensures that a certain subset of substrates gets phosphorylated under the right conditions and in a correct tempo-spacial pattern. Mitogenic and growth inhibitory signals use both positive and negative regulators to regulate Cdk activity. Givent that these regulators control cell proliferation, it is not surprising that the positive regulators often have been found to be proto-oncogenes while negative regulators often are tumour suppressors. The cell controls the activity of Cdk in a variety of ways, although not by regulating the levels of the Cdk proteins, since these levels are stable during the cell cycle. The monomeric form of these kinases is inactive and an absolute requirement for their activation is the association with a subunit from a protein family called cyclins. The name cyclin refers to the fact that if one measures the levels of these proteins over several round of cell division, they re-occur in a cyclic manner. This is because that, due to transcriptional induction and controlled protein degradation, cyclins only occur at specific stages of the cell cycle. Cyclins are suggested to regulate the substrate specificity of the Cdks. During one round of cell cycle, different cyclins-Cdk combinations occur at different time

points and locations, resulting in a specific subset of substrates being phosphorylated (120). The activity of the cyclin-Cdk can also be more rapidly regulated by phosphorylations. For example, the ATP binding site of the Cdk subunit can be blocked by inhibitory phosphorylations and this inhibition can be released by the Cdc25-family of phosphatases (44, 100). The Cdk can also be positively regulated by phosphorylation by Cdk activating kinases (CAKs). For example can the CAK cyclin H-Cdk7 activate Cdk1 (36, 37) .In addition, the association with Cdk inhibitors (CKI) of the CIP/KIP or INK protein families inhibit cyclin-Cdk activity. Thus, the activity of a Cdk depends on the relative activity, levels and localisation of its regulators. The protein levels of these regulators are determined by regulated transcription, translation and proteasomal protein degradation. The two large ubiquitin-ligase complexes which controls the levels of many regulator are the Skp/Cullin/F-box (SCF) complex and the anaphase promoting complex (APC). These complexes excert direct control over cell cycle progression (135).

The complex control of Cdk activity result in the timely correct, sequential activation of different Cdk activities that is the main driving force of the cell cycle. Mammalian cells express both multiple Cdks and cyclins (120). As mentioned above, this enforces substrate specificity but it also allows a larger variety of ways to regulate cell cycle progression.

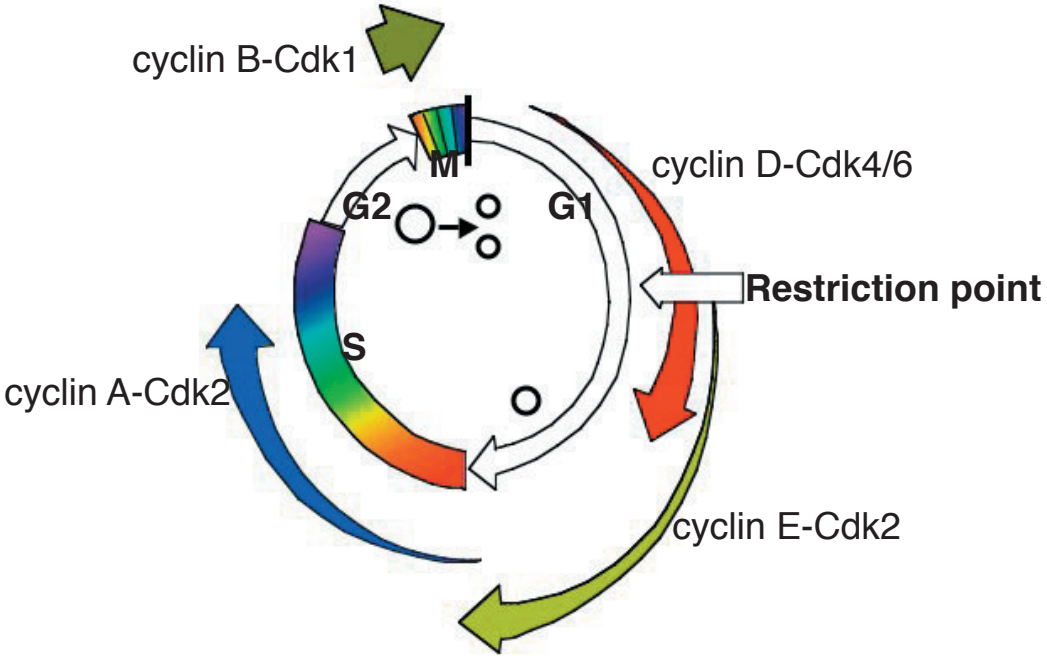

Figure 1. **The basic cell cycle machinery in mammalian cells**
Different combinations of cyclins and Cdks over time result in a specific timing of distinct Cdk activities. Since each cyclin-Cdk recognises a certain subset of substrates, this results in different substrates being phosphorylated at specific time points. Thereby, each cyclin-Cdk activity controls the induction of the following cyclin-cdk activity. The phosphorylation of specific substrates at a correct time constitutes the mechanism that drives the cell through the different cell cycle transitions (based on Tessema, M. et al 2004).

G1-phase progression

Stimuli from the extracellular environment, such as growth factors and cell anchorage can cause a quiescent cell to start to proliferate. These stimuli initiate signalling cascades, which results in activation of cyclin D-Cdk4/6. Cyclin D-Cdk4/6 kinase activities will thereafter remain active as long as mitogens are present (120). The signals from extracellular factors can stimulate cyclin D-associated kinase activity on many levels, as by inducing cyclin D gene transcription, cyclin D mRNA translation and stability, cyclin D protein stability, cyclin D-Cdk assembly and translocation. In addition, mitogens also regulate the association of Cdk4/6 family to CKI (120). The cyclin D-Cdk complex stimulates G1-phase progression in two ways. First, the cyclin D-Cdk4/6 phosphorylate members of the

retinoblastoma tumour suppressor protein family, pRb, p107 and p130. These phosphorylations inhibit the function of pRb proteins (48). All members of this protein family inhibit cell proliferation, and the importance of this function is highlighted by the observation that pRb is functionally inactive in most tumour cells (41). Because unphosphorylated, active pRb family members represses E2F-dependent transcription, these phosphorylations result in the release of a subset of E2F-dependent transcriptional programs. These include transcription from the cyclin E and cyclin A genes (20, 104). In addition, the E2F transcriptional programs also activates genes whose products function in DNA metabolism and replication, as Thymidine synthase, MCM helicase and CDC6 (19). Second, increase of cyclin D-Cdk4/6 complexes also sequesters $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ of the CIP/KIP family of Cdk inhibitors. These are potent inhibitors of Cdk2 but not of cyclin D-Cdk4 kinase activity. Actually, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ has been shown to assemble or increase the stability, and thus activity, of the cyclin D-Cdk4/6 complexes(109). Thus, both cyclin D-Cdk4/6 phosphorylation of pRb members and sequestration of Cdk inhibitors induce the activity of cyclin E-Cdk2. Once cyclin D-dependent kinase activity has been achieved and pRb inactivated, the cell do not longer depend upon growth factor stimuli to enter S-phase (73). Therefore, this shift has been suggested to be the mechanism at the Restriction point underlying the transition from a extracellular-dependent to an extracellular-independent stage (16). The activity of cyclin E-Cdk2 is induced by cyclin D-associated kinase activities and increases therefore a couple of hours after cyclin D-Cdk4/6 activity. The cyclin E-Cdk2 activity phosphorylates the pRb protein at additional sites leading to the complete inactivation of its transcriptional suppression, and thereby releases transcription of additional S-phase inducing genes. pRb also associate with histone deacetylase and other chromatin remodelling proteins. These interactions are also altered by the phosphorylations mentioned, and are likely be important for S-phase induction, although the exact mechanism remains unknown (48). Cyclin E-Cdk2 activity is periodic and peaks at the G1/S-phase transition (64) (34). Cyclin E-Cdk2 phosphorylates $p27^{KIP1}$, which targets this CKI for ubiquitination and protasomal degradation by the SCF-Skp2 ubiquitin ligase (11). Thereby, cyclin E-Cdk2 activity induces a positive feed back loop, increasing cyclin E-Cdk2 kinase activity even further. The G1-phase kinase activities are required for loading MCM helicase onto chromosomal DNA and the formation of complexes involved in DNA replication (19). This presented model of G1-phase progression explains how events during the G1-phase prepare the cells to start DNA-replication.

S-phase progression

CyclinE-Cdk2 activity is abruptly shut off in the early S-phase (34, 64). This is due to the phosphorylation, and thereby targeting, of cyclin E for ubiquitin-based proteolysis. This targeting of cyclin E for destruction actually relies on cyclin E-Cdk2 (11). Cyclin A-Cdk2 activity is detectable first at the G1/S-phase border. This activity increases during DNA replication and declines at early mitosis when cyclin A is degraded(132). During S-phase, this kinase activity phosphorylates substrates that start DNA replication from pre-assembeled replication initiation complexes and prevent re-replication(130)

G2-phase progression

After completion of DNA replication, some time passes before the start of mitosis. This period is called the G2-phase. During this period cyclin A-dependent kinase activity increases, and peaks after the entry into mitosis (132). Cyclin A-kinase activity is required and also time limiting for G2- to M-phase transition (43). It is therefore likely that cyclin A-dependent kinase activity coordinates the end of S-phase with the onset of mitosis. During late G2-phase, cells are preparing for mitosis by inducing kinase activities of cyclin A-Cdk2, polo-like kinase (Plk)1 and Aurora B (94).These kinase activities are important for mitosis. For example, Plk1 is required for the formation of a correct mitotic microtuli-based spindle and Aurora B phosphorylates histone H3 therby mediating chromosome condensation. (2, 125, 52) . Cyclin B1 starts to accumulate in the cytoplasm during the S- and G2-phases (60). Although it is unclear why it takes some time after the DNA replication has ended before mitosis starts, it is most likely due to a control for replication errors ensuring that the DNA-replication has resulted in DNA of the correct quality and quantity. Because this is the only cell cycle period when the two, newly synthesised, identical copies of DNA are aligned, this period may allow the repair system to use the

copies as templates for the homologous repair system (125, 50). The DNA-control during this period, the G2-checkpoint, ensures that only cells with a correct genome may enter mitosis and give rise to two daugther cells. This control is therefore crucial for the integrity of the genomic material. The G2-phase control of genomic integrity may be so important that all cell cycle components mediating the G2- to M-phase transition all are substrates this control, which is further described below (125).

Mitosis and cytokinesis

The last phase of the cell cycle, the M-phase, is the phase exhibiting the most apparent visual changes of cell morphology. The different stages of mitosis are traditionally defined based on the visible changes of chromosomes and the dynamic, bipolar microtubule based spindle that function in chromosome segregation and cell division. Mitosis starts when the chromatin condenses (115). Cyclin B1 translocates rapidly to the nucleus at the G2-/M-phase border, resulting in nuclear cyclin B-Cdk1 activity. Thus, the first stage of mitosis, Prophase, exhibits condensed chromatin and nuclear cyclin B-Cdk1 activity. Once cyclin B-Cdk1 is activated mitotic entry is beyond recall and the cell will not be able to exit mitosis until this complex is inactivated (94). Nuclear cyclin B-Cdk1 activity triggers important mitotic events, such as the breakdown of the nuclear envelope (116). The disassembly of the nuclear envelope marks the start of the following phase, Promethaphase.

During Promethaphase, the spindle microtubuli enters the area close to the chromosomes and attaches to specific protein structures on the centromeres of the chromosomes, the kinetochores. The chromosomes are thereafter attached to the mitotic spindle and translocated towards the middle of the cell. During this period, the condensed daughter chromosomes remain coupled to each other by securin. A large ubiquitin-ligase complex that mainly functions during mitosis and the exit from mitosis is the anaphase-promoting complex, APC. The substrate specificity of APC is controlled by cofactors, such as Cdc20 (24). The APC-Cdc20 complex targets cyclin A for destruction at the Promethaphase to Methaphase transition (24).

At Metaphase all chromosomes are positioned in the middle of the mitotic spindle, and aligned in the middle of the cell. APC-Cdc20 marks cyclin B and securing for degradation. Degradation of securin results in the separation of the sister chromatides, which marks the onset of Anaphase (24).

During Anaphase, the mitotic spindle starts moving the new daughter chromosome towards the opposite poles of the cell to form two groups of separated chromosomes at the centrioles. The APC change to the cofactor Cdh1 during late Anaphase. This leads to a different subset of substrates being targeted for destruction. APC-Cdh1-mediated proteolysis functions in coordinating late mitotic events. The APC-Cdh1 mediates for example the degradation of Plk, an event that has to take place in order for cytokinesis to occur (24). A newly formed nuclear envelope is thereafter assembled around each group of chromosomes.

This is followed by the last stage of mitosis, Telophase, during which the chromosomes start to decondense.

Anaphase onset is followed by initiation of cytokinesis. First, a set of bundled microtubuli, the central spindle, is formed in the middle of the two centrioles, and thereby determines the division plane in the cell (47) (89). Thereafter, actin filaments are assembled at the becoming cleavage furrow, either by being transported there or by new actin polymerisation. An acto-myosin contractile ring is further assembled and contraction of this ring result in the initiation of cleavage furrow ingression. Factors involved in the mechanism of furrow ingression include the small GTPase RhoA, Rho kinase (ROCK), Citron kinase and myosin light chain kinase (MLCK) (47, 103). A possible model of furrow initiation could be that once activated by the Rho guanine nucleotide exchange factor Pebble, RhoA activates effectors known to be required for cytokinesis. These are the formins, which nucleate unbranched actin and ROCK that phosphorylates the myosin II regulatory light chain activating myosin II-based actin contraction. Because the plasma membrane is attached to the underlying acto-myosin network in the cleavage furrow, it is deformed during furrow ingression. The insertion of membrane vesicles forming new plasma membrane is therefore required for cytokinesis. This occurs via a mechanism that is separate from the actomyosin contractile ring (103, 47). The ingression of the cleavage furrow result in a tightly constricted waist between the two forming daughter cells.

The compressed central spindle forms the bridge between the cells, the midbody. In the last step in cytokinesis, the midbody either deattaches from one daughter cell or is parted in the middle. This, results in the final separation of the daughter cells (47) (93). This event is thought to depend upon a mictotubule-based transport to the furrow that resembles exocytosis. How this final separation of the daughter cells and the closure of plasmamembrane occurs is not known in detail, but the midbody helps in concentrating regulatory proteins, such as the GTP-ase activating protein (GAP) Rho GAP and Aurora B. Plk1 and Citron kinase also play key roles in cytokinesis since lack of these kinases results in defects in cytokinesis (125, 103) .

Cell cycle checkpoints

In order to assure that only genetically intact cells are allowed to divide and give rise to daughter cells, there are several controls of DNA integrity during one cell cycle round. These DNA-controls are often referred to as ”Checkpoints”. The word checkpoint is however also sometimes used to describe a molecular event allowing the cell to pass from one cell cycle phase to the next. If the genetic material is not of correct quality, quantity, structure or localisation these DNA –checkpoint mechanisms halts cell cycle progression until the genomic integrity is restored. If the repair fails, these mechanisms lead to controlled cell death, apoptosis, or permanent cell cycle arrest. DNA damage or arrest of replication fork leads to the activation of the ataxia telangiectasia mutated (ATM) and AMT- and Rad3-related (ATR) kinases. This causes a cell cycle arrest. The substrates of these kinases include $p21^{CIP1/WAF1}$, cyclin E-Cdk2, CDC25- phosphatases, cohesin, Plk and cyclin B-Cdk1 (11). These DNA quality checkpoints can be activated throughout the G1-, S- and G2-phases of the cell cycle.

A checkpoint in the M-phase is the “Mitotic spindle checkpoint” in methaphase, which ensures that all chromosomes are correctly aligned and attached to the mitotic spindle before allowing chromosomal separation in anaphase.

There is an ongoing debate on whether there exists a ”Tetraploidy checkpoint” in the G1-phase of the cell cycle or not. This checkpoint would ensure that only cells that correctly passed through cytokinesis and are diploid are allowed to continue progression through the G1- and S-phases of the cell cycle. Indeed, cells which fail cytokinesis due to treatment with the actin-polymerisation antagonist Cytochalasin will arrest in the following G1-phase. This arrest is dependent on functional p53 (3). But if cells are treated with a low concentration of Cytochalasin, cytokinesis will fail, but the two formed nuclei will nevertheless continue into a new round of DNA replication (123). This implies that there exist no tetraploidy checkpoint, and that tetraploidy as such not is the cause of the observed G1-phase arrest observed in the above experiments.

An oversimplified model of cell proliferation?

This above classical model of cell cycle progression is a useful model when investigating the mechanisms underlying cell proliferation. However, a number of observations indicate that this model might be oversimplified. For example, pRb rarely is found on E2F promoters during the G1-phase of the cell cycle (8). Also, a mouse strain lacking all three D-type cyclins develops to midgestation without any obvious pathological phenotype. The levels of cyclin E/A, Cdk4/6/2 were unaffected in cells from this mouse strain and cyclin Cdk2 activity not decreased by the cyclin D loss (67). In addition, animals lacking either Cdk4 or 6 are viable, although an elimination of both these Cdks result in a phenotype similar to the elimination of all cyclin D genes(75). This implies that complete elimination of cyclin D function does not compromise the cell cycle *per se* and raises questions on the prevalent dogma that is outlined above.

The major issue regarding the current model of the cell cycle machinery is nevertheless results obtained by the Cdk2 null mice. These mice are viable and, except for sterility, free of all pathological conditions. One can imagine several explanations for the above results obtained using null mice. For example, some other components during mouse embryogenesis and life could functionally compensate for the loss of the eliminated gene product. Some experimental data suggest for example that cyclin E- or cyclin A- associated kinase activities can compensate for the loss of cyclin D during mouse embryogenesis (110). Less studied or yet unknown kinases, Cdks and cyclins could compensate

for the eliminated molecules. If put in an evolutionary perspective, it is also possible that remaining ancient mechanisms of DNA replication and mitosis may function as back up mechanisms during mouse development.

Components of cell anchorage to the extracellular matrix

Components and functions of the extracellular matrix

Cells produce, assemble and secrete insoluble fibrous proteins, soluble adhesive glycoproteins, proteoglycans and hyaluronic acid (15, 72). These compontents form a network between the cells called the extracellular matrix (ECM). There are different types of ECM. These include the intersititial stroma, bone, elastic connective tissue, cartilage and the thin basal lamina that lies under all epithelial sheets (72). The ECM provides scaffolding support that helps the cells to organise into tissue and keeps the integrity of the organs. Cellular binding to ECM binding also regulates important cellular functions, such as cell polarity, adhesion, motility, differentiation, survival and proliferation. The ECM has therefore a major impact on cell behaviour, both during embryonic development and in the growing and adult body (72). The ECM can also indirectly regulate cell processes by binding factors, such as growth factors, or by forming gels of varying charge and thereby control the cellular access of different molecules (72) .

The fibrous proteins are mainly the insoluble collagens, while the adhesive glycoproteins consist of soluble multi-adhesive proteins such as fibronectin, laminin and vitronectin (72). Proteoglycans are composed by unbranched, negatively charged polysaccaride chains, glucosaminoglycans, which are covalently linked to a protein core (131, 72).

Another important component of the ECM is the very large glycosaminoglycan hyaluronic acid (HA) that consists of 50.000 repeats of a single sugar molecule (72). HA is the only extracellular oligosaccaride that is not covalently attached to a protein. If stretched out from one end to another, one HA molecule would be 2 cm long (72). However, the very negatively charged HA curls up, attracts water, and thereby forms gels that resist compression. HA is therefore a major component in for example the joins. HA can also form complexes with proteoglycans, and such complexes are part of many extracellular matrices, for example cartilage. HA is also a major component of the extracellular matrix surrounding migrating and proliferating cells, particulary in embryonic tissues (72).

Structure and function of integrins

The main extracellular matrix receptors on the cell surface are the integrins (53). Integrins constitute a large protein family of transmembrane, heterodimeric proteins. They are an essential link between the ECM and the actin cytoskeleton and are named after their ability to integrate and transmit structural order between these two systems. While the amino-terminal extracellular domain of integrins is large (700-1100 amino acid residues), the integrins transmembrane region and the C-terminal cytoplasmic tail are small (30-50 amino acid residues). Integrins are composed of one α– and one β–subunit that are non-covalently linked by their extracellular regions. The 18 α and 8 β integrin subunits occur in at least 24 different combinations that mediate cell-cell and cell-matrix interactions in animals (53). Compared to cell surface growth factors receptors, integrins show relatively low affinity for their ligands (72). One integrin dimer may bind several matrix proteins and every individual matrix proteins can be bound by several integrins. Although their functions are overlapping, each integrin heterodimer have distinct ligand-binding and signalling properties. Integrins can transmit mechanical and biochemical signals in either direction across the cell membrane. Upon ligation of extracellular matrix proteins, the integrins transmit signals from the ECM into the cell via so-called “outside-in signalling”. This involves clustering of integrins and gathering of signalling and cytoskeletal complexes that promotes actin filament assembly. The reorganisation of the cytoskeleton into stress fibers causes increased integrin clustering, which results in increased ECM binding and also integrin-dependent organisation of the extracellular matrix. The extracellular binding activity of integrins can be regulated from the

inside of the cell. This process is called “inside-out signalling”. During this process, modifications of the cytoplasmic part of the integrin result in conformational changes in the extracellular domain and ligand binding. Integrins transduces in these ways signals and structural order across the plasma membrane. Therefore, integrins serve as a link between the extracellular matrix and the cytoskeleton (45, 53, 63)

The short intracellular domain of integrins does not harbour any enzymatic activity. Signals from the integrins are therefore transduced via adaptor proteins that connect the integrins to enzymatic signalling components, such as kinases and GTPases. The recruitment of different proteins results in the formation of protein complexes at the place where integrins bind the extracellular matrix. These integrin and integrin-linked components form “cell adhesion complexes”. When these structures acquire kinase activity and induce actin stress fiber formation, they mature into protein clusters called “focal adhesion complexes”. More than 50 different proteins localize to focal adhesions. The combination of ECM-ligand and integrin governs which proteins that will be recruited to the intracellular tail (49). Integrin signalling depends, for example, upon the kinase activity as well as the protein adaptor functions of the non-receptor tyrosine kinases focal adhesion kinase (FAK) and Src. There are numerous mechanisms of integrin “outside-in” signalling, and I will here focus on some well-studied pathways that include proteins related to this thesis. Although integrin signalling can take place independent of FAK, a common characteristic of the activation of intracellular signals by integrins is that they recruit FAK. FAK can be regulated by phosphorylation, which both regulates FAK kinase activity and its function as a protein scaffold (49). Notable is that only integrins anchored to a solid substratum, and not integrin-engagement in the surface of cells in suspension triggers FAK phosphorylation. Phosphorylated FAK then recruits the Src-family kinases(22). These kinases can thereafter, via a variety of components, activate different compontents such as extracellular signal mediated kinases (ERKs)/mitogen-activated protein kinases (MAPK) and the Rac and Cdc42 effector p21-activated kinase (PAK) (26,101). Src-family kinases can phosphorylate and activate PAK either directly or by signalling via Rac. Alternatively, FAK can directly recruit Grb/Sos and thereby activate the Ras/Raf/MEK-cascade, which activate ERK/MAPK. (49, 114) . Celladhesions structures of cells cultured in 3D model systems have low FAK activity (133), implying that FAK-independent integrin signalling may be of more important under these conditions.

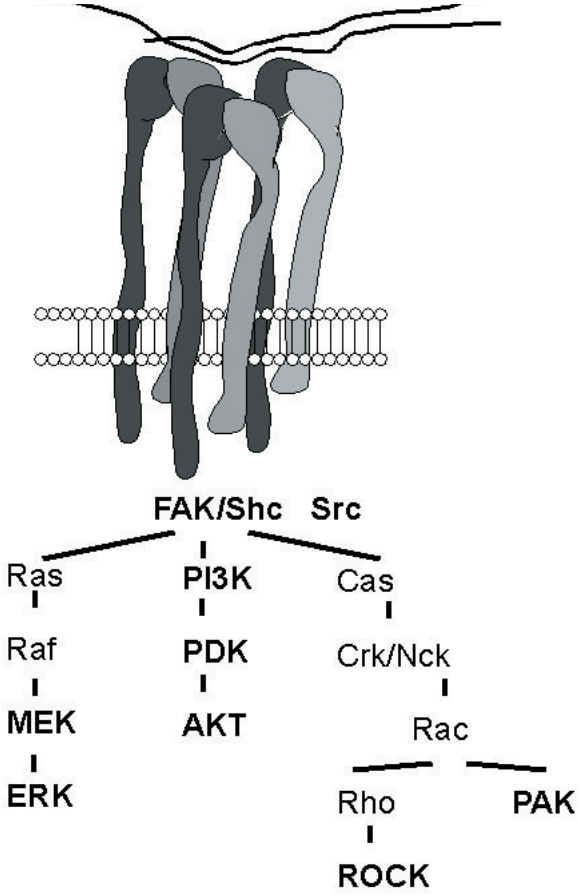

Figure 2. **Examples of integrin sigalling** Upon ligation to the extracellular matrix, integrins initiate signalling that can be both dependent or independent of FAK and/or SFK. These signals may result in the activation of kinases such as MAPK, PI3K and PAK. The well studied integrin-dependent signals shown in this figure regulate cell proliferation (based on Stupack, D.G. and Cheresh, D.A. 2002).

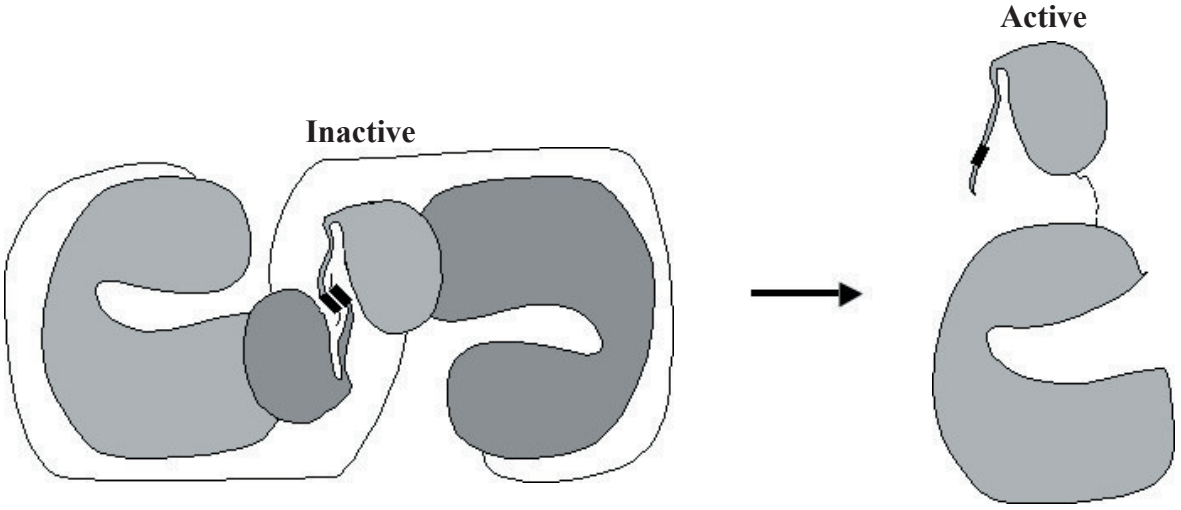

Figure 3. **Model of PAK activation**
Schematic image of PAK activation. GTP-bound Cdc42 or Rac bind the CRIB sequence within the autoregulatory region. Thereby the PAK-dimer is dissociated and kinase domains exposed, (based on Lei, M. et al 2000)

p21-activated kinases (PAK) are evolutionary conserved serin/threonin protein kinases. PAKs contain an amino-terminal regulatory domain and a highly conserved carboxy-terminal catalytic domain. PAKs are activated by the binding of GTP-loaded small Rho family GTPases. Although PAK initially was found when searching for proteins interacting with the p21 RhoA, and is also named thereafter, PAK is activated by the binding of activated Rac and Cdc42 but not by Rho. The small GTPases Cdc42 and Rac bind to the CRIB domain in the amino-terminal part of the protein. The six found PAKs have, based on sequence homology, been divided into two subgroups, PAK 1-3 and PAK 4-6 (18). The function and regulation differ significantly between these groups, and I will focus only on PAK 1-3. Cell anchorage control PAK 1-3 activity via Rac and Cdc42 (9, 32). An autoinhibitory region in the amino-terminal part of the protein controls the basal kinase activity of PAK 1-3. PAK1 exists as homodimer and the autoregulatory kinase inhibitory domain (KID) of one PAK1 molecule inhibits the kinase domain of the other *in trans* (90). PAK1 have also been reported to be activated in GTPase independent ways, such as by recruitment to activated tyrosine kinase receptors at the plasma membrane, by sphingolipids and, PAK 2, by caspase-dependent proteolysis (18). Nevetheless, the common theme for activation of PAK1 is that the homodimer is disrupted and the autoinhibitory domain, KID, is displaced. This leaves the kinase domain of PAK in an open conformation, accessible to substrates.
Activated PAK 1 is localised to focal adhesions and to the leading edge of migrating cells (18, 143). These are regions with ongoing actin cytoskeletal remodelling. Overexpression of PAK1 induces significant cytoskeletal and morphological effects, like filopodias, lamellopodia, dissolution of stress fibers and focal adhesion complexes as well as retraction of the trailing edge in migrating cells (76, 77, 106, 142). Therefore, PAK1 probably play critical physiological roles in controlling cytoskeletal organisation. Several lines of evidence suggest a role for PAK1 in cell migration. The role of PAK in cytoskeletal reorganisation, and also the finding that PAK 1 may activate FAK (59), could explain the importance of PAK1 for cell migration.
An important mediator of PAK1 function to control the cytoskeleton is the LIM kinase. PAK 1 binds, phosphorylates, and thus activates LIM kinase. The only known substrate for the LIM serine kinase is cofilin, which binds to and catalyze F-actin depolymerisation and severing. When phosphorylated by LIM kinase, cofilin can no longer bind F-actin and this results in increased amount of F-actin filaments. LIM kinase can also be phosphorylated on the same residues by Rho kinase (ROCK). A critical event in the regulation of acto-myosin contractility is the phosphorylation of the regulatory myosin

light chain (R-MLC) at Ser 19 and (Thr 18) by Ca2+-dependent myosin light chain kinase (MLCK). These phosphorylations increase the intrinsic ATP-ase activity of the myosin and thereby stimulate contractility. Also PAK1 and 2 can phosphorylate the critical Ser 19 residue in the R-MLC, and may thus also contribute to acto-myosin contractility. In contrast to this function, PAK1 and 2 can also inhibit this contractility by phosphorylating MLCK at an inhibitory site. Another PAK1 substrate that modulates the actin cytoskeleton is filamin A, which induces cross-linking of actin filaments.
It is also possible that filamin A, by binding also Rac and Cdc42 can act as a platform and assemble components for activation of PAK1 by small GTPases (18).

The extracellular matrix receptor CD44; structure and function

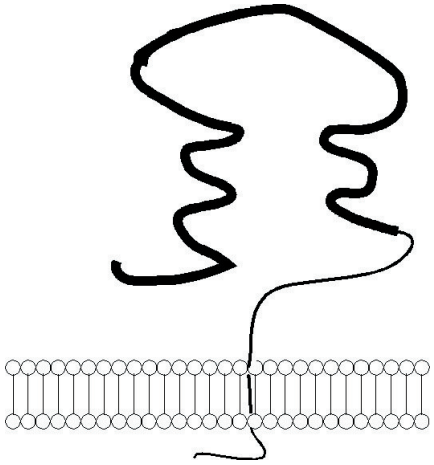

Figure 4. **Schematic image of standard CD44 protein structure**
The cell surface adhesion protein CD44 binds the extracellular matrix component hyaluronic acid by its extracellular globular domain. The hyaluronic-binding domain of CD44 is marked by a thick line. The globular domain is anchored to a transmembrane domain by a highly variable stem region which contains sites for heparan sulphate as well as a putative cleavage site. These regions are followed by a short cytoplasmic tail (based on Ponta, H. et al 2003).

The primary receptor for hyaluronic acid is the transmembrane receptor CD44 (4), and several CD44 receptors can bind on the large HA molecule at the same time. CD44 can link the cell to ECM by binding to HA, mediate cell-cell interactions and function in cell migration and HA-metabolism. The structure and functions of the members of the CD44 protein family are very diverse. This is due to different splicing and post-translational modifications, causing many variants of CD44. The extracellular part of CD44 consists of a conserved amino-terminal HA-binding globular domain and a highly variable stem structure. The smallest CD44 variant is called the standard isoform. The stem-stucture of this variant is 46 amino acid residues long. This stem region contains a putative cleavage site. These extracellular domains are followed by a transmembrane domain and a carboxy-terminal cytoplasmic-tail region (96). CD44 can regulate cellular functions by different mechanisms. The extracellular domain of CD44 can bind the fibroblast growth factor (FGF) via heparan sulphate side chain on its alternatively spliced exons. This function of CD44 has been shown to be required for FGF-8 to stimulate the proliferation of nearby cell (107). CD44 can also regulate cellular signalling by functioning as a co-receptor for receptor tyrosine kinases, such as Met and FGF Receptor 4 (96). It is likely that this function can depend upon CD44 bringing the receptor and ligand together. CD44 itself may also transduce signals through proteins linked to the cytoplasmic tail. The intracellular tail of CD44 can also be linked to and organize the cortical actin cytoskeleton. In addition, the CD44 cytoplasmic tail has phosphorylation sites that can affect HA-dependent cell migration (91). Taken together, this indicates that signal transduction takes place in pre-formed units, in which CD44 bring ligand, receptor, enzymes and substrates together. (61, 96, 122) .

Proteoglycans; structure and function
There are two cell surface localised proteoglycan subfamilies that link cells to the ECM. These proteoglycans are connected to the plasmamembrane by the core protein having either a transmembrane region (syndecan) or by being anchored by a glycosylphosphatidylinositol (GPI) linkage (glypican) (71). The main cell surface proteoglycan type, syndecans, has been shown to be required for integrin-mediated assembly of focal adhesions (27). In addition to binding ECM components, and thus mediating cell anchorage, proteoglycans can also impact cellular signalling cascades such as FGF2 signalling. This function of proteoglycans is probably mediated by the proteoglycan retaining and stabilizing extracellular ligand, and bringing the ligand and receptor close to each other (71, 27).

Cell anchorage controls the cell cycle and tumour progression

Cell anchorage to the extracellular matrix controls proliferation of tissue cells and the abrogation of this control is a critical step in cellular transformation. Cell anchorage-independent growth is an essential oncogenic propriety of cancer cells.

The extracellular matrix and cancer
Degradation and remodelling of the ECM is required for many steps during the development of a malignant tumour, such as in the growth of the primary tumour, metastasis and the formation of new blood vessels, angiogenesis. This degradation is in part carried out by zink-dependent matrix metalloproteinases (MMP) (126). Furthermore, the alteration of ECM receptors function and their downstream signalling molecules are involved and required for cellular tranformation and tumour progression (29, 70, 13)

Cell anchorage in different cell cycle stages

Cell anchorage controls the G1-phase
The extracellular matrix-dependent control of cell cycle progression must be altered in order to transform a normal cell into a tumour cell. G1-phase progression and S-phase entry is dependent upon the sequential activation of cyclin D-, cyclin E- and cyclin A-associated kinase activities. Both integrin-mediated cell anchorage and growth factor activation of receptor tyrosin kinases is required for the induction of cyclin D kinase activity. Integrin- and growth factor signalling converge on different levels to induce G1-phase progression (29). Integrins exerts a strict spacial and positional control on the action of receptor tyrosine kinases at the plasma membrane, and is also required for growth factor activation of ERK and to induce transcription of D-type cyclins (99). Growth factors and cell anchorage to the ECM controls G1-phase progression also in more separate ways. For example can cell anchorage control translation of cyclin D transcripts while growth factors regulate cyclin D protein stability (6). Another way for these extracellular factors to control G1-phase progression, is by regulating the activity of the substrate-recognition subunit Skp2 of the SCF ubiquitin ligase. Growth factors and anchorage are required for the Skp2 protein stability and Skp2 gene transcription, respectively, (23, 129). SCF (Skp2) ubiquitin ligase can control Cdk2 and Cdk1 activities through its principal downstream effector, the CKI $p27^{KIP1}$(88). In addition, without any control upon cyclin D, cyclin E, or pRb-phosphorylations, anchorage controls cyclin A expression in a rat kidney cell line and in Harvey (H) -Ras transformed rat ER1-2 fibroblasts (62, 144). An intact G1-phase control by cell anchorage is a crucial part of the cellular defence against uncontrolled cellular proliferation and the development and progression of tumours.

Cell anchorage, mitosis and cytokinesis
During one cell cycle, the apparently most dramatical changes in cellular morphology and cell

anchorage take place during mitosis and cytokinesis. The onset of mitosis is accompanied by cell rounding due to cellular de-adhesion, cortical retraction and increased cortical rigidity. At this stage, components of focal adhesions such as FAK, paxillin and p130CAS becomes heavily serine/threonine phosphorylated and the focal adhesions are disassembled. Thus, the signal transduction from focal adhesions are inactivated (134, 74). The cell rounding is most prominent at promethaphase. Thereafter, the cell re-establishes contacts with the underlying matrix resulting in increased cell anchorage and cell spreading. The re-adhesion is accompanied by dephosphorylation of FAK (40) and its mitosis-specific serine phosphorylation is reversed during post-mitotic spreading. So far, only one report suggests a role for integrins in cytokinesis. In this study the authors observed that integrin beta1 localises to the cleavage furrow of dividing chondrocytes and that the integrin beta1-deficient chondrocytes have defect cytokinesis. However, this phenotype was not the observed in integrin beta 1-deficient ES cells, fibroblasts, keratinocytes or hemapoetic cells. (7). Normal tissue cells need to alter the regular control of cell cycle by cell anchorage in order to be able to transform into a tumour cell. If cell anchorage would control the cell cycle also during mitosis and cytokinesis, the abrogation of such control would be a key step in tumourigenesis.

Cell anchorage in the establishment of a primary tumour

In addition to its role in the creation of a tumour cell, the ECM also plays crucial roles in the establishment of a primary tumour. Cell needs to be able to evade programmed cells death, apoptosis (92), in order to create a primary tumour. Cell-ECM attachment can give cells the capacity to avoid apoptosis, and ECM may thereby promote primary tumour formation. In addition, primary tumour establishment also requires that transformed cells have the capacity to stimulate new blood vessels formation by sprouting from existing blood vessels, angiogenesis. The new blood vessels will supply the tumour cells with growth factors, nutrients and oxygen and are required for a tumour to grow larger than approximately 1 mm^3. Tumour cells initiate angiogenesis by secreting soluble factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). These factors activate blood vessel endothelial cells to produce and secrete MMPs that will break down the ECM. As a result, the endothelial cells will start to invade into the surrounding tissue. As the cells migrate they also start to proliferate and divide. The remodelling of the ECM is a pre-requisite for this phase in angiogenesis. Thereafter, the endothelial cells organize into hollow tubes that later differentiate into a network of new blood vessels. Also, this maturation stage is dependent upon ECM, since it requires the formation of a new basement membrane (38).

Cell anchorage in metastasis

Cancer spreads by metastasis, which is the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade and grow in normal tissues elsewhere. It is mainly this ability to spread to other tissues and organs that makes cancer a potentially life-threatening disease (112). Remodelling of the extracellular matrix and changes in cellular anchorage plays key roles in the process of metastasis. A tumour cell can metastasize in different ways. It can invade a lymph or blood vessel, circulate in the lymphatic or blood stream, attach to the vessel wall and invade a normal surrounding tissue elsewhere. It can also invade metastasize via invading the surrounding tissue or cavities. Tumour cells need to de-attach, degrade and migrate through the surrounding ECM during all types of metastazing. Thus, the process of metastasing involves and dependent upon different cell-ECM interactions. Constant remodelling of the cell-ECM interactions is thus needed throughout metastasis (49).

PAKs and cancer

There are several lines of evidence linking PAK to cellular mechanisms important for cellular transformation and tumour progression. PAK1 activity has been indicated to stimulate, and be required for, high migration and invasiveness of breast cancer cell lines (124). Also, NIH3T3 cells become more motile when expressing constitutively activated PAK1, and expression of dominant negative PAK1 disturbs NIH3T3 cell migration. Possibly, PAK could regulate the direction of cell migration

via inhibition of MLCK and thereby suppression of R-MLC phosphorylation and contractility in the leading edge of the cell (18). The lack of PAK activity on the sides and at the rear of the cell could allow retraction of these regions (18). This model of PAK1 role in cell migration is consistent with the fact that regulation of actin cytoskeleton by Cdc42/Rac and RhoA counteracts in migrating cells. While Cdc42/Rac are important at the leading lamella of cells, Rho activity functions at rear of the cell (18).

In addition to migration, PAK may also function in cell survival and proliferation. Several lines of evidence suggest a role for PAK in controlling cell cycle progression. First, hyperactive PAK1 and 2 have been found in highly proliferative breast cancer cell lines (80). Second, the most commonly activated human oncogene, Ras, induce PAK1 kinase function, which is required for Ras-induced transformation of Rat-1 cells as well as for the proliferation of different transformed cells. However, PAK1 kinase function is not required for Ras-induced transformation of NIH3T3 cells (117, 118). Also, PAK1 has been reported to induce cellular transformation and also to cooperate with Ras in inducing anchorage-independent growth (18, 117, 124). Furthermore, constitutive active PAK1 induced mitotic defects in breast cancer cell lines (124). In addition, activated PAK1 is localized at the contractile ring during cytokinesis and the yeast PAK homologues in budding yeast, Saccharomyces cerevisiae, are essential for mitosis and cytokinesis (28, 127). PAK1 is important for the microtubule organisation that is needed in the leading edge of a migrating cell as well as during cytokinesis. Activated PAK1 also localises to centrosomes and can induce centrosomal abnormalities (69, 83). In mitosis, cyclin B-Cdk1 phosphorylates PAK1 leading to less stable microtubuli (18). This phosphorylation of PAK1 does not change the kinase activity of PAK1, but rather modifies PAK protein-protein interaction and function as a protein adaptor or scaffold (121). Such kinase-independent functions of PAK have been described but this role of PAK is less well studied.

CD44 and cancer

The standard isoform of the ECM-receptor CD44 can induce mestatasis (65) and variants of CD44 are aberrantly expressed in many tumours. One variant of CD44 is also involved in tumour metastasis and the expression of certain CD44 variants predicts poor prognosis of colorectal carcinoma (128). These observations suggest that CD44 can act like an oncogene and promote tumour progression. However, it should be noted that the CD44-variants that are aberrantly found in many tumour could also be a result of the genomic instability of tumour cells. In contrast to the notion that CD44 is an oncogene, several lines of evidence indicate that CD44, if overexpressed, can inhibit tumour growth. First, it has been shown that when injected in mice, mouse fibrosarcoma cells overexpressing the standard isoform of CD44 have a longer latency time than the parental fibrosarcoma cell line. Once the CD44 overexpressing tumour cells have established a palpable tumour, the CD44 overexpression was downregulated (65). Also, by comparing subcutaneous tumour growth of transformed CD44 mouse fibroblasts with the same cells where the standard isoform of CD44 is reintroduced, it became clear that the CD44 can significantly reduce primary tumour growth (102). In addition, in some tumour types the absence of CD44 correlates with transformation and poor prognosis (31, 111). These observations indicate that CD44 also can act as a tumour suppressor.

In addition to its function in cell adhesion, CD44 can also affect different cell function by bringing enzymes and their substrates together at the plasma membrane. For example, it has been shown that CD44 can recruit MMP9 to the cell surface, and that MMP9 thereafter can induce collagen degradation and also tumour-cell invasion (138). Other reports suggest that CD44 may recruit MMP7 to the plasma membrane and that this can suppress apoptosis in tumour cells (139).

Aims of the study

The overall aim of this study was to elucidate mechanisms of cell anchorage component-mediated control of cell cycle progression.

Specific aims:

• To determine at which cell cycle stage cell anchorage controls cell cycle progression and reveal functional molecular mechanisms for this control (papers I and II).

•To elucidate the role of the kinase inhibitory domain of the anchorage dependent p21-activated kinase1 (PAK1) in cell cycle control (paper III).

•To examine if recombinant hyaluronic acid binding domain of CD44 may affect in endothelial cell cycle progression (paper IV).

Evaluation of materials and methods

The methods below are, like all methods, dependent upon well-matched controls in order to be conclusive. When analysing the results, both the requirements for the experimental design as well as the sensitivity and specificity of the method of analysis should be confirmed. Several methods complement each other and thus collectively give more reliable results.
Therefore, we aimed throughout the studies in this thesis to use several methods when investigating an important phenomenon.

Human contra mouse cells in cell proliferation studies.
Rodent cells immortalise and transform spontaneously (51). Only culturing primary mouse embryonic fibroblasts and dividing them 1:3 each third day, will over time result in an immortalised cell line, This cell line is named 3T3-cells. It has long been established that primary rodent cells can be transformed by two oncogenic ”hits”, like ectopic c-myc expression and constitutively active Harvey-Ras (H-Ras), or the early region of simean virus 40 (SV40T) together with activated H-Ras . In contrast, primary human cells are relatively resistant to transformation. For example, introduction of c-myc and active H-Ras only causes human cells to become irreversibly growth arrested. Although the reason for the difference between human and mouse cell’s resistance to transformation is unclear it is evident that human cells differs from mouse cells in that the human cell cycle must harbour additional controls of proliferation (33, 51). Thus, if the ultimate aim for the studies of cell proliferation is to understand human tumourigenesis, it is advantageous to use human cells for the study. When mouse cells are used, this difference in cell proliferation control should be kept in mind. The advantage of using 3T3 cell lines is that they, in contrast to primary human cells, are easy to transfect. This makes it possible to observe the cell cycle effect by the gene of interest. Human primary cells can be immortalised by overexpression of hTERT (17). This human cell-line can be useful for cell cycle studies in that it is transfectable and more resistant to transformation than mouse cells. However, it should be considered that expression of hTERT may disturb the serum-dependent control of the cell cycle.

Cells suitable for studies of anchorage-dependent growth
While epithelial and endothelial cells undergo apoptosis when denied of cell anchorage, fibroblasts arrest in the cell cycle. In order to study cell cycle control by cell anchorage or PAK1, we therefore choose fibroblasts of different origin in these studies.

Why synchronise cells at a certain cell cycle stage?
One can examine progression through a certain cell cycle stage in individual cells or by studying a large cell population. If a whole cell population is studied, a synchronisation of the cell population and a following release into the part of the cell cycle of interest is required for several reasons. First, this lowers the background of cell in other phases, which ensures that the exact cell cycle stage of interest is under investigation. In addition, low background and a large cell population are required in order analyse the cells with biochemical methods, such as assaying for cyclin-dependent kinase activities. We synchronised cells in the G0/G1-phase by growth factor depletion and/or contact inhibition. Cells arrested at the G1/S-phase border were obtained by treating cells with the DNA polymerase-inhibitor Aphidicoline. Because Promethaphase-cells loose cell anchorage and round up, cells synchronised in this phase were obtained by mitotic shake off. In some cases cells were treated with the microtubule polymerisation inhibitor nocodazol that arrest cells in promethaphase prior to shake off. Nevertheless, a significant disadvantage with cell synchronisation is that this can affect cells so that their cell cycle differs from an undisturbed cell cycle. This is especially notable when synchronising cells with the use of drugs. A cell released from quiescence undertakes a G1-phase that differs from the G1-phase of a cell that just has completed a previous round of cell division. Most normal cells rest in a quiescent stage and can be stimulated to proliferate in the presence of growth factors. Therefore, it can be presumed that

the G1-phase that follows G0/G1-phase release most correctly reflects the G1-phase of a normal cell. Most likely, studies of these two types of G1-phase progression both provide important information on mechanisms underlying oncogenic transformation. Nevertheless, it is important to clearly mark what type of G1-phase progression that has been examined. The clear advantage of studying cell cycle progression of single cells, is that they do not need to be synchronised and thus are undisturbed in this regard. However, it should be noted that using a large cell population provide the possibility to study cell cycle progression with a large variety of methods, while the methods available to study cell cycle progression in a single or a few cells are limited. In addition, using a cell population consisting of ten thousands of cells, rules out the possibility that non-representative characteristics of individual cell will impact the results.

Gene transfer by transfection

We used both transiently transfected and stably transfected cell lines in these studies. When comparing these strategies for genetransfer and expression, it is clear that both has advantages and disadvantages. A clear advantage of transient transfection is that a cell population expressing the protein of interest is obtained just a few days after transfection, while it takes many weeks to select for and to expand stably transfected clones. Transient transfections introduce a high number of gene copies into the cell, while stable transfection only results in one or a few copies of the gene of interest being introduced. As as consequence, transient transfection normally gives higher expression levels than stable transfection. After transient transfection, the expression usually rises to a detectable level within 24 h, peaks at 36 h and then declines to undetectable levels after 72 h. These variations of expression levels over time, as well as the fact that the transfection-efficiency can vary excessively between different DNA-constructs used as well as between experiments, result in lower reproducibility than stable transfection. It can also be difficult to combine the protocol for transient transfection with the experimental setup for cell cycle studies. This is due to the fact that cells should normally be exponentially growing, or be grown at certain density, prior to transfection. This can be difficult to combine with methods used for cell cycle synchronisation. Another advantage of stable transfection is that clones expressing a well-defined level of the gene of interest can be used for long-term functional studies. The protein levels expressed in clones are lower than after transient transfection, and thus better resembles a protein level of an endogeneous protein. A disadvantage of using a stable transfested clone is the possibility that clones can harbour or develop some characteristics, which make it uncertain to refer phenotypes of the clone to the gene of interest. It is, however, difficult to totally control for the possibility that the introduction of the gene, indirectly, over time will result in additional phenotypes. The use of a tet-off system circumvents this problem. This is due to that cells containing a tetracyclin sensitive transactivator together with the gene of interest under the control of this regulator does not express any protein when grown in the presence of tetracyclin. An additional advantage with the use of the tet-off system is that it includes the possibility of dose-dependent regulation of protein expression. However, it is crucial that the tet-controlled transactivator is silent when cells are grown in the precence of tetracyclin, and that the serum used when inducing protein expression totally lack any traces of tetracyclin.

Methods used to distinguish between cell cycle phases

The fact that cells prior to replication contain a 2n genome while cells after replication contains 4n provides the possibility to determine a cell populations distribution in the cell cycle with a stain against total DNA. The amount of DNA can then be determined by analysis by a Flourescence Analysis Cell Sorter (FACS). This is a fast and accurate method which provides the possibility of determination of the fraction of cells in a cell population that are in G0/G1-phase, G2/M-phase and the intermediate S-phase. Another way to study the proliferation status of a cell population is to immunostain for the thymidine-analogue Bromo-deoxyuridine (BrdU). Replicating S-phase cells can then be detected by anti-BrdU immunostaining and analysed in a fluorescence microscope. This provides the possibility to obtain information not only on the S-phase fraction of cells but also of the morphology of the same cells. While, this method indicates the S-phase cells, it does not provide information on the overall cell

cycle distribution of a cell population. We most often used a double staining against both total DNA and anti-BrdU followed by FACS-analysis. This double-stain analysis of FACS-data allows a dot-plot. This provides the possibility to distinguish between G0/G1-phase, S-phase and G2/M-phase of the cell cycle. In contrast to total DNA stain, the double-stain gives the possibility to identify the cell cycle positioning of each single cell analysed. The double-stain also stains specifically for S-phase cells and therefore provide the possibility to distinguished early and late S-phase cells from the adjacent phases. Since the S-phase population after a double-stain only shows actively replicating cells, cells that have arrested during DNA replication can be disregarded.
Condensed chromatin was easily detectable after a total DNA stain using Hoechst or Giemsa by analysis in a in a flourescence or phase-contrast microscope, respectively. Of these methods, Giemsa has the advantage of being a very fast staining. Immunostaining against phosphorylated Histone H3, a component of condensed chromatin, together with PI-stain followed by FACS-analysis allowed a discrimination of the mitotic cell population. There are several advantages of using FACS as compared to manual quantification in a fluorescence miroscope to analyse fluorescent cells. One is that it enables analysis of larger amount of cells and can easily analyse 10.000 individual cells. This provides more statistically significant results. FACS depends to a less extent upon subjective evaluations and is thus a more objective method of analysis. As a consequence, results from FACS are generally more reliable. Nevertheless, FACS can never provide any morphological data as can a microscope, and it is therefore advantageous to use both the above methods when studying the cell cycle distribution of a cell population.

Methods to detect cytokinesis
After fixing cells onto a glass by cytospin, we used several staining methods to detect binuclear cells and cells in cytokinesis. Indicators of cytokinesis are anti-tubulin or anti-aurora B stainings of the midbody during cytokinesis. Therefore, we detected cells in cytokinesis with this these stainings. After a Giemsa staining, chromatin shows as light violet, condensed chromatin appears as dark violet and the cytoplasm light pink. Because the protein dense midbody clearly stained dark purple, we were able to use Giemsa staining to detect cells in cytokinesis. The major benefit of the Giemsa staining it that the time it takes to perform the staining can be counted in minutes, while it takes hours to stain for either anti-tubulin or anti-aurora B, actin and Hoechst. Otherwise, the later stainings gives the most accurate result since it directly identifies specific molecules involved in cytokinesis.

Different ways to detect apoptosis
Effects on apoptosis was determined by FITC-conjugated Annexin-V staining of flipped phosphatidyl serines, followed by FACS-analysis. This provides a marker of an early event in apoptosis. Apoptosis was also FACS-analysed after staining with total DNA content. As cells in late apoptosis contain fragmented DNA, this shows as a sub-Go/G1-phase population. These above methods will also to a certain degree stain necrotic cells. But, since we merely wanted to control that the our cells were not undergoing any kind of cell death, these methods fulfilled our demands.

Immunoblotting versus kinase assays in cell cycle studies
In order to determine the cause of an observed cell cycle arrest, we used immunoblotting and kinase assays to detect protein levels and cyclin-associated kinase activities, respectively. Assaying for cyclin-dependent kinase activity detect the functional activities during the cell cycle. Therefore, while analysis of protein levels only can give an indication of why a cell is arrested in a certain cell cycle stage, analysis of Cdk activities provide a direct functional explanation to a cell cycle arrest. It should be kept in mind, however, that the detection of a specific protein level or enzymatic activity does not necessarily indicate that this activity is of functional importance.

Results and discussion

In order to proliferate, normal tissue cells depend upon the extracellular enviroment. This extracellular control of cell proliferation is defect in tumour cells. The major extracellular factors regulating normal cell proliferation are soluble growth factors and cell anchorage to the surrounding extracellular matrix. In comparison to the growth factors-dependent control on cell proliferation, the nature of the anchorage-dependent control is less well studied. We therefore investigated when and how cell anchorage and some of its components control cell cycle progression.

The control by cell anchorage can be separated from the control by growth factors during the cell cycle G1-phase (paper I)

Growth factors control cell proliferation only in early-to mid G1 phase, before cells pass through the Restriction point. The control of the Restriction point has been suggested to depend upon the tumour suppressor pRb (12, 95) . If cells are denied growth factors after this point, they will replicate their genome, divide independent of growth factors and arrest in the subsequent G1-phase (16). Although not thoroughly investigated, the control on cell proliferation by other extracellular factors, such as cell anchorage, have also been considered to occur during early- to mid G1-phase, through the Restriction point and the pRb-pathway (16, 79).

We investigated the need for serum and cell anchorage in the G1-phase. Thereby, we found that in disagreement with the above doctrine, serum and cell anchorage were required during two overlapping, but distinct, periods in the G1-phase. In accordance with earlier reports, we found that serum was needed until mid G1-phase (57) (140). However, anchorage was required longer, until late G1-phase for the cell to enter S-phase. By investigating the requirements for serum and anchorage during the G1-phase in cells lacking pRb and p107, both members of the pRb-protein family members, we observed that while the serum-dependent control in mid-G1 phase depended upon these pRb family members, the anchorage-dependent control in late G1-phase did not require pRb and p107. This late G1-phase control by cell anchorage was then further characterized. Because cyclin E-associated kinase activity peaks in the late G1-phase, we hypothesized that the anchorage-dependent control on the late G1-phase was mediated by this kinase activity. Indeed, cells lacking pRb and p107 were unable to induce cyclin E-associated kinase activity without cell anchorage. This provides an explanation to why these cells required anchorage in the late G1-phase. Although the protein levels of cyclin E and of the cyclin E-Cdk2 inhibitor $p21^{CIP1/WAF1}$ were similar in cells with and without anchorage, the protein level of $p27^{KIP1}$ was significantly induced only in cells lacking cell anchorage. These results imply that cell anchorage may control the late G1-phase through $p27^{KIP1}$ dependent control of cyclin E-Cdk2 activity.

Anchorage is needed to induce D-type cyclins and cyclin D associated kinase complexes in early-mid G1-phase. This stimulates the subsequent activation of cyclin E/Cdk2. Therefore, it could be argued that the anchorage-dependent control of cyclin E-Cdk2 activity that we observe in late G1-phase is just a consequence of anchorage dependent control on cyclin D-associated kinase complexes. However, the observation that pRb/p107-deficient cells lacked detectable amounts of cyclin D-dependent kinase activity and the reports that cells lacking pRb do not require cyclin D (73) makes this possibility improbable. The major downstream mediator of the ubiquitin ligase SCF(Skp2) is $p27^{KIP}$ (88). Cell anchorage is needed to accumlulate the Skp2 protein (23). It is thus possible that loss of cell anchorage inactivates Skp2-mediated $p27^{KIP1}$degradation, results accumulation of $p27^{KIP1}$, and consequently in inhibition of cyclin E-kinase activity. Furthermore, the observed cooperation of Skp2 with cyclin D in rescuing proliferation in suspended cells (23), suggests a role for Skp2 in an anchorage-dependent control that is separate from the control in the early/mid-G1-phase. Our observations functionally separated the serum-dependent control from the anchorage-dependent control during the G1-phase. Our results also provided functional evidence showing that the control of the G1-phase by serum acts through the retinoblastoma protein family pathways, which is consistent with other reports

(12, 95). In addition to the control in the early/mid G1-phase, cell anchorage was also found to control later G1-phase events independently of cyclin D-associated kinase activity and pRb family members. We hypothesize that the cell anchorage-dependent control in late G1-phase acts through downregulation of cyclin E/Cdk2 activity, possibly by Skp2-degradation and a subsequent accumulation of $p27^{KIP1}$.

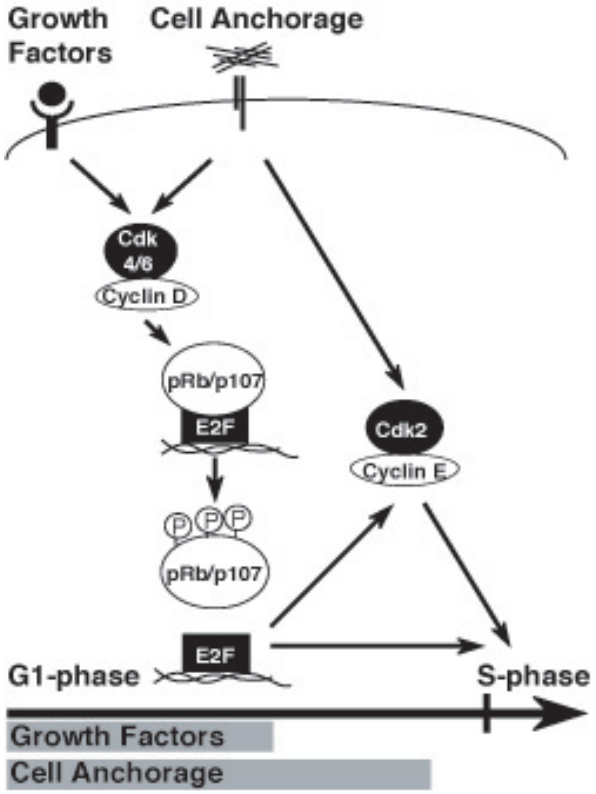

Figure 5. **Hypothetical model of growth factor- and cell anchorage-dependent control of the cell cycle G1-phase**
We found that while primary fibroblasts need serum until mid-G1-phase, they require cell anchorage until late G1-phase (horizontal grey bars). We suggest that serum and anchorage control G1-phase progression until the inactivation of pRb family members pRb and p107 in mid G1-phase. Thereafter, progression through the late part of G1-phase is controlled by anchorage, via a control that is independent of normal serum control, cyclin D-associated kinase activity, pRb and p107. This control may instead be executed by cyclin E-associated kinase activity. (This figure is reprinted from Gad, A. et al 2004, with permission from the publisher)

Cell anchorage controls the cell cycle during cytokinesis (paper II)

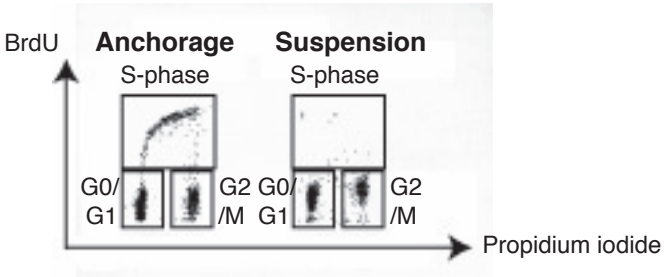

Figure 6. **Anchorage-dependent control of cell cycle progression**
We cultured normal fibroblasts for 24h, either with anchorage or in suspension. Thereafter, we analysed the cell cycle distribution by flow cytometry. We obseved that cells lacking anchorage arrested not only in G0/ G1-phase but also during G2- or M- phases of the cell cycle (reprinted from paper II).

Our finding that cell anchorage controls the G1-phase also after the previously described point in early/ mid G1-phase, encouraged us to search for possibly unknown controls by cell anchorage also after the G1-phase. While studying this, we could observe that S-phase progression was independent of cell anchorage. This implies that once replication has started, it proceeds and terminates independently

of cell anchorage. Furthermore, progression through the G2-phase and the different stages of mitosis was independent of cell anchorage. We thereafter examined if cell anchorage was required for the final cell division, cytokinesis. Importantly, the final cell division separating the two daughter cells did not occur without cell anchorage. Unexpectedly, the initiation of the cleavage furrow constriction took place, but the final completion of cell division separating the two daughter cells did not occur without cell anchorage. We further found that even though cell anchorage is essential for cytokinesis, cell spreading as such is not. Our results further indicated that both various types of integrins as well as integrin-independent anchorage to a positively charged poly-L-lysine-surface can provide the cell anchorage required for cytokinesis. Because the major ligand for poly-L-lysine has been reported to be proteoglycans (56, 68), this suggest that cell anchorage by proteoglycans may be sufficient for cytokinesis. In agreement with this, heparin coating of the poly-L-lysin surfaces blocked cell anchorage and cytokinesis. We also observed that overexpression of activated H-Ras enables the cells to complete cytokinesis in the absence of anchorage and ROCK activity.

A few earlier observations indicate that cytokinesis could depend upon the two daughter cells migrating away from each other during cytokinesis (21) (81) (25). Such mechanism of cytokinesis should require integrin-mediated cell anchorage. However, our results indicated that integrin-independent cell anchorage was sufficient for cytokinesis. In addition, cell spreading was not required for cytokinesis. We therefore suggest that the anchorage during cytokinesis is not required to allow extension and migration of the poles of the dividing cell but rather to regulate cleavage furrow ingression directly. Actin polymerisation and actin-myosin based contraction are both required for cytokinesis (78, 113). Therefore, we wondered if cell anchorage may control cytokinesis via actin polymerisation or by acto-myosin contractility. In order to investigate in cell anchorage control cytokinesis via actin polymerisation we used Cytochalasin B that blocks de novo actin polymerisation without interfering with already polymerised actin. We could observe that although H-Ras overexpressing cells had lost the need of anchorage, they still required actin polymerisation in order to complete cytokinesis. We therefore conclude that the requirement for actin polymerisation during cytokinesis is distinct from the need of anchorage. This notion is also supported by our finding that cell anchorage is not needed for the initiation of cleavage furrow ingression, actin polymerisation has been reported to be crucial for this event. However, we could not exclude the possibility that anchorage controls cytokinesis via actin polymerisation, but at a later stage of cytokinesis. It has previously been reported that cells treated with a low dose of Cytochalasin B initiate cleavage furrow formation but fail during the final abscission in late cytokinesis (123). Therefore, we wondered if anchorage was required for actin polymerisation at this late stage of cytokinesis. We hypothesised that if anchorage was required in order to polymerise actin in late cytokinesis, the H-Ras cells should be less dependent not only of cell anchorage, but also of actin polymerisation during this period. When testing this we could observe that the H-Ras expressing cells were just as sensitive to low doses of cytochalasin B as control cells (data not shown). This may suggests that actin polymerisation during late cytokinesis is required also in cells that divide independently of cell anchorage. Thereby, this could imply that actin polymerisation is not part of the anchorage-dependent mechanism that activated H-Ras can override. This is in agreement with the notion that cell anchorage is not required for actin polymerisation as such. Blebbistatin is an agent that blocks the ATP-ase activity of myosin II and thereby acto-myosin-based contractility. In agreement with previous reports we observed that this activity is required for cytokinesis (113). Three kinases could regulate the regulatory myosin II light chain during cytokinesis. These are MLCK, ROCK and citron kinase (46). Our results indicated that MLCK not is required for cytokinesis, and this pointed to the possibility that either ROCK or citron kinase are required for myosin-mediated contractility in cytokinesis. In agreement to this, blocking ROCK1/2 activity with a ROCK-inhibitor, clearly reduced the ability to complete cytokinesis in untransformed cells. In contrast, the anchorage-independent H-Ras cells were resistant to the ROCK inhibitor, implying that these cells do not require ROCK activity to complete cytokinesis. This suggests that ROCK activity may be a part of the anchorage-dependent control of cytokinesis that can be overcome by activated H-Ras.

Rho is the only member of the Rho-protein family of small GTPases, which is required for cytokinesis in animal cells (55) (46). The Rho effector ROCK is a key molecule in transmitting

signals of mechanical tension from the cell surrounding by phosphorylating a variety of proteins responsible for actin-myosin based contractility (98). One of these substrates is the regulatory myosin light chain that can be phosphorylated at two sites that each results in higher ATP-ase activity and acto-myosin contractility (54). Although ROCK has a higher affinity for one of the sites, highly elevated ROCK activities can phosphorylate both sites (136). Extracellular mechanical force has earlier been shown to activate Rho and lack of tension to a substrate has been shown inhibit Rho activity (87). In the light of our findings, this may imply that the defect cytokinesis that we observed in cells denied of cell anchorage is due to loss of Rho/ROCK activity. Anchorage may be envisioned to control RhoA activity during cytokinesis by regulating $p27^{KIP1}$ protein levels, which has been shown to bind and inhibit RhoA function (5). Another possibility may be that upon anchorage, the major cell surface proteoglycan syndecan binds and activates RhoA (84). Taken together, out observations may indicate that cell anchorage is needed in order to sustain high levels of ROCK activity required for actin-myosin ring contraction. Nevertheless, cell anchorage-dependent ROCK activity could not be required for the initiation, but for the completion of acto-myosin ring contraction. In a very preliminary and hypothetical model one may envision that a basal, anchorage independent, level of ROCK activity phosphorylates the myosin regulatory light chain at one phosphorylation site, and that this is sufficient for cleavage furrow initiation. At a later stage of cleavage furrow contraction, it may be possible that anchorage is needed to obtain high local ROCK activity to phosphorylate also the additional phosphorylation site in R-MLC. The resulting double phosphorylation could possibly result in increased contractility that may be required for the final part of cleavage furrow ingression.

The kinase inhibitory domain of PAK1 control G1-phase progression (paper III)

The focal adhesion kinase PAK1 is an effector of the small GTPases Rac and Cdc42. PAK1 is important for cell motility, cytokinesis and has been implicated in cellular tranformation and tumour progression (18). Growth factors and cell anchorage regulate Rac activity, which is required and time limiting for the induction of cyclin D (105), and thus also for cell cycle progression. Therefore, we hypothesised that PAK1 might be involved in cell anchorage-dependent control of the cell cycle G1-phase. Supporting this hypothesis, we found that a dominant negative variant of PAK1 repressed progression through the G1-phase of the cell cycle. Somewhat more puzzling was that also wt PAK1 inhibited the G1-phase. Nevertheless, a constitutively active variant of PAK1 did not show any effect on the cell cycle. Further suggesting a role for PAK in cell cycle control, we observed that the PAK kinase inhibitory domain (KID) inhibited G1-phase progression and also induction of cyclin D1 and D2. However, to our surprise also a KID-variant defective in PAK binding caused G1-phase arrest. This implies that the cell cycle inhibition by KID is not dependent upon its ability to inhibit PAK1-kinase activity, but rather on its interaction with another molecule. KID induced cell cycle arrest was not reversed by co-expression of a constitutively kinase-active version of PAK1, further implying that KID may inhibit cell proliferation by a PAK-kinase activity independent mechanism.

We hypothesize that the PAK kinase inhibitory domain interacts with and inhibits component(s) that are crucial for cell cycle progression. This function of the PAK kinase inhibitory domain is observed using an isolated domain. Could the kinase inhibitory domain of the intact endogenous PAK have the same function? Such a function of PAK would be separate from PAK kinase activity. There are few but convincing reports showing that PAK1 has other functions in addition to acting as a kinase. For example, PAK1 can function as a protein scaffold to control cellular events, such as the stability of microtubule during mitosis (121, 18) . In addition, the cytoskeletal and morphological effects observed when overexpressing PAK1 can be both dependent and independent of its kinase activity (30) (42). Our results with the KID domain and the fact that both dominant negative and wild type PAK could inhibit the G1-phase, could be explained if one envisions that the kinase inhibitory domain of both these proteins inhibits G1-phase progression. However, the constitutively active form of PAK1 did not affect the cell cycle. This may imply that the kinase inhibitory domain is not as accessible in this PAK1 variant as in the other variants. A difference between the constitutively active variant and the dominant negative and wild type variants of PAK1 is that the constitutively active PAK1 is autophosphorylated. Thus, it is possible that this autophosphorylation could render the kinase inhibitory

domain unaccessible to a potential cell cycle regulator. According to this model, PAK1 may regulate cell cycle progression in two separate ways. PAK could either promote cell proliferation by its function as a kinase and by the binding of the PAK1-kinase inhibitory domain to a component required for cell proliferation. In this scenario, dominant negative PAK1 could inhibit cell proliferation by both these pathways, while wild type PAK1 only inhibits the kinase-independent function. Such dual functions of PAK1 may therefore explain why we observed a stronger cell cycle inhibition by the dominant negative variant of PAK1 than by the wild type PAK1. Our finding that the KID function is independent of PAK1 kinase activity points towards the importance of better understanding the kinase-independent functions of PAK1. Our results imply that a kinase-independent function of PAK1 can control cellular proliferation and suggest that this uncharacterised function should be further studied.

The hyaluronic acid binding domain of CD44 controls endothelial cell cycle progression, angiogenesis and tumorigenesis (paper IV)

CD44 has been suggested to promote tumour angiogenesis, tumour growth and metastasis. However, overepression of soluble CD44 makes tumour cells less vascularised and inhibits tumour growth (138) (137) and overexpression of CD44 in tumour cells result in a delay in tumour formation and metastasis (65). We therefore proposed that a purified recombinant hyaluronic acid binding domain (HABD) of CD44 may be able to inhibit angiogenesis and tumour growth by disturbing the CD44 binding to HA. When investigating this hypothesis we found that recombinant CD44-HABD, even at low doses, significantly inhibited tumour vascularisation and tumour growth. Surprisingly, also a CD44-HABD variant containing three mutations that all are reported or predicted to render the HABD domain deficient in HA-binding gave a similar inhibition of angiogenesis and tumour growth. We also observed that CD44-HABD as well as the variant deficient in HA-binding specifically inhibited proliferation of normal endothelial cell. The CD44-HABD variants were not able to inhibit the proliferation of normal fibroblasts, epithelial cells or tumour cells of various origins. We did not observe any increased apoptosis of cells treated with the recombinant proteins, and therefore suggest that recombinant CD44-HABD induces a cell cycle arrest.

What could be the mechanism underlying the ability for recombinant CD44-HABD to inhibit endothelial cell proliferation? Clearly, a defect in the ability for the cell to bind to HA is not the underlying reason for this inhibition. HA-independent functions of CD44 have been reported earlier. CD44 can be released from the cell surface by proteolytical cleavage and incorporated into the extracellular matrix (85, 86). It is therefore possible that CD44 can affect ECM organisation as well as the access to growth factors by cells, and thereby consequently inhibit cell proliferation. However, since we observed that the recombinant proteins directly bind to the surface of endothelial cells, it seems unlikely that the inhibition of CD44-HABD on endothelial cell proliferation should be due to a direct effect on the ECM. Our results indicate that the CD44-HABD binds a molecule present on the endothelial cell surface and thereby affects endothelial cell proliferation. It is plausible that CD44-HABD can bind proteins regulating cell surface receptors signalling. However, such interactions are shown to be dependent upon post-translational modifications of CD44 alternatively spliced exons that are not present in our recombinantly expressed HABD. CD44 has been suggested to stimulate angiogenesis by recruiting MMP9, which can release VEGF from the ECM depot (138, 14). It could thus be envisioned that the recombinant proteins inhibit cell proliferation and angiogenesis by disturbing this function of CD44. Nevertheless, since our recombinant CD44-HABD triple mutant cannot bind MMP9, we can exclude this possibility. One possible scenario is that recombinant CD44-HABD binds an unknown receptor selectivley present on endothelial cells, interferes with this receptor's signalling and thereby inhibits cell proliferation. It has been predicted that CD44-HABD binds carbohydrates other than HA such as sialyl Lewis X-containing saccarides (10, 66). Injection of anti-sialyl Lewis X-containing saccarides antibodies into extensively vascularised mice tumours significantly decreased angiogenesis and tumour growth (119). Thus, interference by HABD in the function of these saccarides may thus affect endothelial cell proliferation. Regardless of the mechanism, the recombinantly expressed CD44-HABD can inhibit endothelial cell proliferation and angiogenesis and might therefore potentially be used to treat angiogenesis-dependent pathological states, such as cancer.

Conclusions

The overall aim of this Ph.D project was to elucidate how cell anchorage and components of cell anchorage control cell cycle progression. The conclusions can be summarized as follows:

Control by cell anchorage can be separated from the control by growth factors during the cell cycle G1-phase (paper I)

- The normal control by serum on G1-phase progression is abolished in cells lacking the pRb-family members pRb and p107.
- Anchorage-dependent control in late G1-phase is independent of pRb and p107, and therefore separable from serum dependent control.
- The cell anchorage-dependent control in late G1-phase may act via $p27^{KIP1}$-mediated inhibition of cyclin E-Cdk2 activity.

Cell anchorage controls the cell cycle during cytokinesis (paper II)

- Progression through S-phase, G2-phase, the stages of mitosis and the initiation of cytokinesis is independent of cell anchorage.
- Cell anchorage is required for the completion of cleavage furrow ingression.
- Cells require cell anchorage but not cell spreading for cytokinesis.
- Integrin-mediated cell anchorage is not required for cytokinesis .
- Both integrin-mediated cell anchorage and integrin-independent cell anchorage by charge allow cytokinesis.
- Oncogenic H-Ras abrogate the need for anchorage during cytokinesis.
- Oncogenic H-Ras abolish the need for ROCK activity during cytokinesis.

PAK1 and the kinase inhibitory domain of PAK1 control G1-phase progression (paper III)

- Dominant negative PAK1 inhibits cell cycle progression.
- Expression of the PAK1 kinase-inhibitory domain (KID) induces G1-phase arrest.
- KID induced cell cycle arrest depends on the integrity of KID, but is independent of its inhibition of PAK1 kinase activity.
- KID does not act upstream of PAK1 signalling.
- Cells expressing KID fail to induce cyclin D1 and D2.

The hyaluronic acid binding domain of CD44 controls endothelial cell cycle progression (paper IV)

- The HA-binding domain of CD44 selectivity inhibits endothelial cell proliferation, independent of its HA-binding properties.

Relevance and perspectives

Cell cycle control by components of cell anchorage

Compared to growth factor control of cell proliferation, the control by anchorage on cell proliferation is less well studied. The results presented in this thesis underscore the importance of cell anchorage in the normal control of cell proliferation. This emphasizes the importance of anchorage-dependent control in the protection against pathologies caused by defect cell proliferation, such as tumours.

Cell anchorage in the cellular defence against tumorigenesis

We found that cell anchorage exerts several controls of cell cycle progression that occur after and independent of the serum-dependent control. This is in contrast with the widespread view that all extracellular factors only control cell cycle progression at the Restriction point in the G1-phase. Our findings demonstrate that the current model of cell cycle progression, and in particular the idea of the Restriction point, should be modified to allow a better understanding of the mechanisms governing cell proliferation. The observation that cell anchorage controls cellular proliferation at additional cell cycle stages as compared to growth factors, implies that anchorage exerts a more extensive control than growth factors on cell cycle progression. Why is it so? It is plausible that the cell cycle control of soluble growth factors and the controls by cell to ECM anchorage have evolved at least partially independently during evolution. Does this finding reflect that it is more critical for a multicellular organism that proliferation only takes place within correct extracellular matrix environment than in the presence of growth factors? Or may proliferation of a certain subset of cells be more easily controlled by the use of anchorage-dependency than by that of soluble growth factors? It could be presumed that in the first multicellular organisms and in the slug phase of today's cellular slime mould, *Dictyostelium discoideum*, anchorage to an extracellular matrix is a more specific way to control proliferation of a subset of cells than soluble and diffusible growth factors. Also in the human body, an ECM might more specifically control that cell proliferation only occurs at the right place, as compared to soluble growth factors, which may diffuse throughout the tissue. In this way, the ECM could provide a more specific control for which cells that are allowed to proliferate as compared to growth factors.

Our results showing that the kinase inhibitory domain (KID) of the focal adhesion localized kinase PAK1 inhibits cell proliferation in different tumour cell types suggest that the PAK-KID domain could potentially be used to develop a therapy for cancer. The PAK-KID is a widely used reagent to study PAK function. The finding that KID also functions independently of PAK kinase activity suggests that much precautions must be taken using KID to study PAK kinase function. It also points to the importance of understanding the kinase-independent functions of PAK1, such as its scaffolding function. PAK1 is linked both to cellular transformation and tumour metastasis. Therefore, more knowledge about PAK1 can provide information that may contribute to identifying targets that can improve diagnosis and/or therapies for cancer.

A domain of a cell adhesion molecule can inhibit angiogenesis and thereby tumour growth

Presently, there are numerous anti-angiogenic substances and almost two dozens of different substances are being tested as anti-tumour therapies in cancer patients. These substances are commonly grouped into three different classes, based on their mechanism of action. First, there is a group of molecules that prevent bFGF and VEGF from activating endothelial cells. Second, there is a group that by inducing apoptosis, directly inhibits the growth of the endothelial cell. A third group of angiogenesis inhibitors is directed against MMPs. Our finding shows that a domain of a cell adhesion molecule can inhibit cell cycle progression of endothelial cells, angiogenesis and tumourigenesis. Thereby, we have identified a new fourth type of angiogenesis inhibitors. Endostatin is another protein-based anti-angiogenic factor that in clinical trials has been indicated to be beneficial as an anti-cancer treatment (39). Because the CD44-HABD inhibited tumourigenesis at doses 20 times lower than those used for inhibiting tumour

growth in mice with Endostatin (82), the CD44-HABD inhibitor may have the potential to be even more efficient in anti-tumour treatment than Endostatin. In addition to cancer therapy, the inhibitor has may also have a potential for development of therapy for other pathological states that depend upon angiogenesis, such as adult blindness and various forms of chronical inflammation (1).

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