DOCKING PROTEINS $\text{P}_{130}^{\text{CAS}}$ AND $\text{P}_{120}^{\text{CBL}}$ IN INTEGRIN AND GROWTH FACTOR RECEPTOR SIGNALLING

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Abstract

Adhesive interactions between cells and extracellular matrix proteins play a vital role in biological processes such as cell proliferation, differentiation and survival. Integrons comprise a major family of cell surface receptors that mediate these interactions. Integrin engagement triggers adhesion-dependent intracellular signalling cascades that include the phosphorylation of tyrosines in intracellular signalling proteins. Integrin-dependent signals act in concert with signals from growth factors and other signalling receptors. The objective of this thesis was to study how cell adhesion and growth factors interact with intracellular components to regulate cell behavior in normal and transformed cells.

One of the main proteins phosphorylated following integrin ligation in several different cell types is the docking protein p130Cas (Cas), which is tyrosine phosphorylated after stimulation of cells with low concentrations of epidermal growth factor (EGF). Tyrosine-phosphorylated Cas associates with an adapter protein c-Crk, the main binding protein for Cas, suggesting a novel role for EGF in Cas signalling. The interaction of cells with a variety of agonists such as growth factors and integrin ligation results in stimulation of mitogen-activated protein kinases (MAPKs), which control the expression of genes important for many cell functions. Expression of Cas and Crk induces activation of C-Jun N-terminal kinases (JNKs), which are members of MAPK family. JNK activation induced by integrin ligand binding is blocked by the expression of a dominant-negative mutant of Cas or Crk demonstrating an important role for the Cas-Crk complex in integrin-mediated JNK activation.

The proto-oncogene product p120Cbl (Cbl) was identified as the main tyrosine-phosphorylated protein following integrin ligation in hematopoietic cells of myeloid lineage. Tyrosine-phosphorylated Cbl interacts with and activates other signalling proteins, such as Src tyrosine kinase and phosphatidylinositol 3'-kinase (PI 3-kinase), thereby mediating adhesion-dependent signals in hematopoietic cells. Unlike the cellular Cbl, the transforming mutants of Cbl were tyrosine-phosphorylated in an adhesion-independent manner and interacted with and activated signalling molecules both in suspended and in adherent cells. Further, the oncogenic forms of Cbl induced anchorage-independent but serum-dependent proliferation of cells. These results support the view that transformation by Cbl results from constitutive activation of integrin-dependent rather than growth factor-dependent signalling events.

Keywords: cell adhesion, extracellular matrix, tyrosine phosphorylation, oncogene
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Oulu, June 15th, 1999
To my late mother
Abbreviations

BSA bovine serum albumin
CSF-1 colony stimulating factor 1
C-terminus carboxy-terminus
DMEM Dulbecco’s modified Eagles medium
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
EGTA ethyleneglycol-bis(β-aminoethyl ether)tetraacetic acid
FAK focal adhesion kinase
FCS fetal calf serum
GST glutathione S-transferase
HA hemagglutinin
HRPO horseradish peroxidase
JNK c-Jun N-terminal kinase
MAPK mitogen activated protein kinase
MHC major histocompatibility complex
NGF nerve growth factor
N-terminus amino-terminus
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PDGF platelet-derived growth factor
PI phosphatidylinositol
PMSF phenylmethylsulphonylfluoride
RIPA radioimmune precipitation assay
SH2 and SH3 Src homology 2 and 3
SDS sodium dodecyl sulphate
TRITC carboxytetramethylrhodamine isothiocyanate
X any amino acid
List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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1. Introduction

Cell-extracellular matrix (ECM) interactions play an important role in numerous biological processes, including cell survival, growth, differentiation, migration, inflammatory responses, platelet aggregation, tissue repair and tumour invasion (Ruoslahti 1991, Hynes 1992). The major group of proteins mediating these interactions are a family of cell surface receptors known as integrins, named for their role in integrating the intracellular cytoskeleton with the extracellular matrix. Integrins were discovered in the mid-1980s when it was realized that several cell adhesion proteins had related structures and activities, including platelet adhesion protein gpIIb/IIa, several chicken adhesion proteins, a group of lymphocyte adhesion proteins, the VLA family of cell surface antigens, and receptors for fibronectin and vitronectin. Since then, the importance of integrins in cell biology and medicine has made them a focus of intense research.

It is now clear that integrins act not only as structural links between cells and the ECM, but also as active signalling receptors. Integrin clustering triggers activation of several adhesion-dependent intracellular signalling pathways, including activation of protein tyrosine kinases (PTKs) and several serine/threonine kinases, and changes in intracellular pH and calcium, membrane potential and lipid metabolism. It is known that aberrations in these pathways can lead to pathological disorders. Integrin-dependent signals co-ordinate with signals from growth factor and other signalling receptors so that cell adhesion and other factors together regulate complex biological processes.

The aim of the present research was to study integrin signalling events in fibroblasts and hematopoietic cells, with an emphasis on adhesion-dependent tyrosine phosphorylation events. More specifically, the main focus was on two signalling molecules, p130Cas (Cas), a docking protein previously implicated in integrin signalling in various cell types, and p120Cbl (Cbl), a proto-oncogene product, which was identified in the present studies as the main protein tyrosine-phosphorylated following integrin ligation in cells of the myeloid lineage.
2. Review of the Literature

2.1. Integrins

2.1.1. Integrin family of cell adhesion receptors

Integrins are a family of transmembrane proteins, which link the intracellular cytoskeletal elements with extracellular matrix proteins or with counterreceptors in neighbouring cells (Hynes 1987, Ruoslahti & Pierschbacher 1987). Integrins are heterodimeric proteins composed of α and β subunits that can pair to form more than 20 receptor heterodimers (for a review, see Hynes 1992). Today, at least eight β subunits and 17 α subunits are known which could in theory combine to form more than 100 integrin heterodimers. However, pairing of the subunits appears to be much more restricted; some α subunits pair with only a single β subunit, whereas other α subunits pair with several different β subunits (Hynes 1992). The mature α subunit is composed of an N-terminal glycosylated extracellular domain of about 1000 amino acids in length, which is disulphide-linked to a hydrophobic membrane-spanning domain, followed by a short C-terminal tail of 40-60 residues. α subunits carry several divalent cation-binding sites in the extracellular domain. In addition, at least eight of the currently known integrin α subunits (α1, α2, α10, αM, αX, αL, αE, αD) contain a 200 residue sequence insertion (I domain) in the divalent cation binding region, which is thought to contribute to the ligand binding (Stewart & Hogg 1996, Camper et al. 1998). β subunits consist of a glycosylated extracellular domain of about 750 residues, a membrane-spanning domain and a short 40-60 residue C-terminal tail, except for β4 integrin in which the cytoplasmic tail is over 1000 residues in length (Hogervorst et al. 1990, Suzuki & Naitoh 1990, Tamura et al. 1990). β subunit extracellular domains contain a cysteine rich region of unknown function believed to be intramolecularly disulphide-bonded (Calvete et al. 1991).

Integrins bind their ligands in a divalent cation-dependent manner through the specific extracellular domain ligand binding sites, formation of which requires both subunits (for a review, see Schwartz et al. 1995). Integrin cytoplasmic tails contain binding sites for intracellular molecules and they are also important in modulating the integrin function and affinity by so called ‘inside-out’ signalling (for reviews, see Ginsberg
et al. 1992, Sastry & Horwitz 1993). More specifically, the cytoplasmic α subunits are thought to be important in the affinity regulation (O'Toole et al. 1994), whereas the β subunit cytoplasmic domains are critical for the binding to cytoskeletal proteins and inducing intracellular signalling (Chen et al. 1995, Lewis & Schwartz 1995). The β subunit cytoplasmic domains are also important for the localisation of integrins to focal adhesions, which are sites of close cell-matrix interactions and integrin signalling (see later) (Ylanne et al. 1993, LaFlamme et al. 1994).

Many of the integrin ligands are extracellular matrix proteins involved in cell-substratum adhesion, such as fibronectin, laminins, various collagens, tenasin, vitronectin and thrombospondin (Hynes 1992). Some cell surface Ig superfamily receptors (Springer 1990, Tian et al 1997), micro-organisms (Isberg & Tran Van Nhieu 1994) and certain plasma proteins (Hynes 1992) serve also as integrin ligands. Table I presents currently known integrins and their ligands (for original references, see reviews above and Hynes & Bader 1997, Camper et al. 1998).

RGD-sequence in fibronectin was the first defined integrin recognition site. This sequence is also present in vitronectin and a variety of other adhesive proteins (for a review, see Ruoslahti 1996). Several other ligand binding sites are now known, most of which share one amino acid, the aspartic acid (or sometimes the closely related glutamic acid) residue. The aspartic acid is thought to be important because of its potential to contribute to divalent cation binding (Pierschbacher & Ruoslahti 1984, Ruoslahti 1996). It has been suggested that during integrin and ligand association, this residue provides a coordination site for transient divalent cation binding (Ruoslahti 1996).

Integrin function is controlled in many ways. For instance, the complement of integrins expressed by different cell types varies greatly; some integrins are clearly cell type specific while others are expressed in a wide variety of cells (Hynes 1992). Integrins are normally expressed and present at the cell surface constitutively, but in many cases need activation for ligand binding. The events inside the cell that regulate integrin activation are known as inside-out signalling, but they are currently poorly understood. For example, αIIbβ3 integrin is activated and binds fibrinogen only during hemostasis (Ginsberg et al. 1992). Similarly, the leukocytes must be activated for β1 (Shimizu et al. 1990) and β2 integrins (Dustin & Springer 1989) to mediate adhesion efficiently. While inside-out signalling is important in modulating integrin function, the events outside the cell modulate cell functions by signalling via integrins. This ‘outside-in’ signalling by integrins is discussed in the following chapter in detail.
Table 1. Integrins and their ligands

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1</td>
<td>collagens, laminins</td>
</tr>
<tr>
<td>α2β1</td>
<td>collagens, laminins</td>
</tr>
<tr>
<td>α3β1</td>
<td>collagens, fibronectin, laminins</td>
</tr>
<tr>
<td>α4β1</td>
<td>fibronectin, VCAM-1</td>
</tr>
<tr>
<td>α5β1</td>
<td>fibronectin</td>
</tr>
<tr>
<td>α6β1</td>
<td>laminins</td>
</tr>
<tr>
<td>α7β1</td>
<td>laminins</td>
</tr>
<tr>
<td>α8β1</td>
<td>fibronectin, vitronectin, tenasin</td>
</tr>
<tr>
<td>α9β1</td>
<td>laminins, collagens, tenasin</td>
</tr>
<tr>
<td>α10β1</td>
<td>collagens</td>
</tr>
<tr>
<td>αvβ1</td>
<td>vitronectin, fibronectin</td>
</tr>
<tr>
<td>αDβ2</td>
<td>ICAM-3</td>
</tr>
<tr>
<td>αLβ2 (LFA-1)</td>
<td>ICAM-1, ICAM-2, ICAM-3, ICAM-5</td>
</tr>
<tr>
<td>αMβ2 (MAC-1)</td>
<td>C3b component of complement, factor X, fibrinogen, ICAM-1</td>
</tr>
<tr>
<td>αXβ2</td>
<td>fibrinogen, C3b component of complement</td>
</tr>
<tr>
<td>αIIβ3</td>
<td>fibronectin, fibrinogen, von Willebrand factor, vitronectin, thrombospondin</td>
</tr>
<tr>
<td>αvβ3</td>
<td>vitronectin, fibrinogen, von Willebrand factor, collagens, thrombospondin, fibronectin, osteopontin</td>
</tr>
<tr>
<td>α6β4</td>
<td>laminins</td>
</tr>
<tr>
<td>αvβ5</td>
<td>vitronectin</td>
</tr>
<tr>
<td>αvβ6</td>
<td>fibronectin, tenasin</td>
</tr>
<tr>
<td>α4β7</td>
<td>fibronectin, VCAM-1</td>
</tr>
<tr>
<td>αEβ7</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>αvβ8</td>
<td>laminins, collagens, fibronectin</td>
</tr>
</tbody>
</table>

2.1.2. Cellular signalling by integrins

The attachment of cells to their surroundings is important in determining cell shape and in maintaining proper cell function and tissue integrity. One of the most important effects of the ECM on cells is the control of cell survival. Some types of normal cells, such as epithelial and endothelial cells, require attachment to a substrate to survive; upon detachment, these cells will rapidly die through programmed cell death, apoptosis (Meredith et al. 1993, Frisch & Francis 1994). This anchorage-dependency manifests
itself in the inability of normal cells to grow on a semisolid media such as soft agar. Other types of normal cells, such as fibroblasts, are less sensitive to the detachment-induced cell death, but nevertheless require anchorage to a solid substratum for cell cycle progression and proliferation (Stoker et al. 1968, Folkman & Moscona 1978). Upon detachment, fibroblasts are arrested in the late G1 phase in the cell cycle and fail to enter the S phase. This is probably due to inhibition of the cyclin E-CDK2 complex, since the expression of CDK2 inhibitors is elevated in suspended cells (Fang et al. 1996). Cyclin A expression is cell adhesion-dependent in fibroblasts suggesting another possible mechanism for cell cycle regulation by cell adhesion (Guadagno et al. 1993).

The extracellular adhesive proteins not only promote cell survival and growth, but also induce cell spreading and migration (Carter 1965, Ali & Hynes 1978). Cells adhere and spread rapidly on an ECM substratum, and when confronted with limiting concentrations of an adhesive protein applied as a gradient on a surface, will migrate towards the higher concentration (Basara et al. 1985).

The ECM and integrins also influence cell functions by more complex mechanisms which involve the regulation of gene expression, differentiation and cellular metabolism. These effects have been seen in many cell types, including mammary epithelial cells (Streuli et al. 1991), skeletal muscle cells (Volk et al. 1990), adipocytes (Spiegelman & Ginty 1983), keratinocytes (Adams & Watt 1989), chondrocytes (Zanetti & Solursh 1984) and osteoclasts (for reviews, see Vaananen & Horton 1995, Rodan & Rodan 1997). The ECM may exert its effect on cells directly by providing an inductive signal (Pennypacker et al. 1979) or by acting as a competence-inducing factor making cells capable of responding to soluble growth factors (Giguere et al. 1982).

Integrins are also important mediators of cell function for circulating blood cells. One of the most thoroughly studied areas of integrin signalling is the regulation of platelet function by integrins. Circulating platelets become activated by contact with collagens and thrombin within the exposed subendothelial matrix in wound areas. Following platelet activation, the αIIbβ3 integrin binds fibrinogen, thereby promoting platelet aggregation. αIIbβ3 ligation activates intracellular signalling cascades, such as cellular protein kinases, and promotes further platelet activation (Ginsberg et al. 1992). The importance of the platelet integrin αIIbβ3 is illustrated in Glanzmann’s thrombasthenia patients who harbor inactivating mutations in the gene for the integrin β3 subunit (for a review, see Ginsberg et al. 1988). As a result, platelets of the patients with this disease are defective in αIIbβ3 mediated fibrinogen binding and aggregation in response to activation, which results in a severe bleeding disorder. Several lines of evidence also underline the importance of integrins in leukocyte function. Most notably, integrins are important for leukocytes to exit the circulation; neutrophils and monocytes need β2 integrin mediated attachment to the endothelial matrix to extravasate at sites of inflammation. In a rare genetic disease known as leukocyte adhesion deficiency or LAD I, mutations in the extracellular domain of the β2 integrin subunit abrogate β2-mediated adhesion (Springer 1990), resulting in defective β2 integrin mediated leukocyte functions, such as adhesion to endothelium, extravasation, chemotaxis and phagocytosis (for a review, see Etzioni 1994). This deficiency renders patients susceptible to recurrent and eventually overwhelming bacterial infections.

The above-mentioned cell biological events are a result of integrin-induced activation of biochemical pathways inside the cell. These include increase in intracellular calcium
concentration, elevation of intracellular pH, changes in Na\(^+\)-K\(^+\) transport and regulation of lipid metabolism through activation of phosphatidylinositol 3'-kinase (PI 3-kinase), phosphatidylinositolphosphate 5'-kinase and phospholipase-C\(\gamma\) (PLC\(\gamma\)) (Schwartz et al. 1995). Cell adhesion is known to activate serine-threonine kinases such as protein kinase C (PKC) (Chun & Jacobson 1992, Vuori & Ruoslahti 1993). Another family of serine-threonine kinases activated by cell adhesion are mitogen activated protein kinases (MAPKs), which control the expression of several genes important for cell function, including proliferation and differentiation. Small GTP-binding proteins of the Ras superfamily, such as Ras, Rac and Cdc42 are involved in initiating a cascade leading to their activation (for reviews, see Davis 1994, Minden & Karin 1997). Extracellular signal-regulated kinases (Erks) phosphorylate and activate transcription factors such as c-fos and Elk-1, and c-Jun N-terminal kinases (JNKs) phosphorylate transcriptional factors including c-Jun, ATF2 and Elk1, thereby increasing their transcriptional activity (Ip & Davis 1998). Integrin-mediated cell adhesion activates Erks (Chen et al. 1994, Schlaepfer et al. 1994), and recently, also JNKs were shown to become activated as a result of the clustering of integrins by beads coated with anti-integrin antibodies (Miyamoto et al. 1995b).

Tyrosine phosphorylation of several signalling proteins residing in cellular sites known as focal adhesions is one of the first events seen after integrin engagement. The importance of this modification has become clear through intensive studies made in the field during the past years and is discussed in more detail below.

### 2.1.3. Focal adhesions and tyrosine phosphorylation events in integrin signalling

Focal adhesions (see Fig. 1.) are sites of a tight structural attachment of the cell membrane to the underlying structure. They are classically found only in cultured cells, but appear to be morphologically and functionally analogous to the sarcolemmal dense plaques of smooth muscle cells, myotendinous junctions and adhesion plaques of activated platelets in vivo (for a review, see Jockusch et al. 1995). ECM proteins such as fibronectin and vitronectin are localised outside of the cell membrane, and a number of proteins which serve to anchor actin to the membrane have been identified at the focal adhesion cytoplasmic face. Integrins are the major transmembrane components that mediate cell attachment to the ECM at focal adhesions (Hynes 1992). Binding of integrins to their ligands leads to clustering of integrins, protein complex formation and actin stress fibre localisation at the cytoplasmic face of the plasma membrane.
Fig. 1. A simplified model of focal adhesion organisation showing putative links from actin stress fibres to the sites of cell-ECM adhesion. PM; plasma membrane, py; phosphorylated tyrosine residue.

Aggregation of integrins is a cornerstone in assembly of focal adhesion; if the aggregation is inhibited, focal adhesion formation is prevented. Formation of focal adhesions involves coordination of several different events, including actin polymerisation, actin stress fibre attachment to the membrane and stress fibre contraction. A model for focal adhesion assembly was recently proposed by Burridge and coworkers (Chrzanowska-Wodnicka & Burridge 1996). In this model, ligand-bound integrins in quiescent cells are linked to actin microfilaments through cytoskeletal structural proteins α-actinin and talin. Following serum stimulation, components of serum such as lysophosphatidic acid would activate the small GTP-binding protein Rho. Active Rho in turn would activate yet-to-be-identified intracellular signalling pathways leading to integrin clustering and focal adhesion formation. In support of this model, inactivation of Rho has been shown to result in focal adhesion disassembly (Ridley & Hall 1992).

Focal adhesions serve not only as a structure for cells to anchor to the ECM but also as an important biochemical signalling site. When integrins are clustered from outside with beads coated with anti-integrin antibodies or integrin ligands, a large number of signalling molecules associate with aggregated integrins (Miyamoto et al. 1995a, Miyamoto et al. 1995b). It is now well understood that assembly of signalling molecules in the focal adhesion cytoplasmic face takes place in a tyrosine phosphorylation-dependent manner and that tyrosine phosphorylation events serve as a means to regulate signalling events inside the cell. Integrins do not have any intrinsic kinase activity, which suggests that an integrin activated tyrosine kinase(s) must be present and active in focal adhesions. Indeed, a novel cytoplasmic tyrosine kinase termed as focal adhesion kinase or FAK (also known as p125FAK) (Hanks et al. 1992, Schaller et al. 1992), which localises to
the focal adhesions, was found to become tyrosine-phosphorylated and activated after integrin ligand binding and clustering (Burridge et al. 1992, Guan & Shalloway 1992, Hanks et al. 1992, Kornberg et al. 1992, Lipfert et al. 1992, Vuori & Ruoslahti 1993). Currently, FAK is considered as one of the key molecules in integrin signal transduction.

2.1.3.1. Focal Adhesion Kinase in integrin signalling

FAK is a 120 kDa cytosolic tyrosine kinase with a central catalytic domain flanked by large N- and C-terminal domains (see Fig. 2.) (Hanks et al. 1992, Schaller et al. 1992). A focal adhesion targeting (FAT) sequence resides in the C-terminus and is both necessary and sufficient for focal adhesion localisation (Hildebrand et al. 1993). The amino acid sequence of FAK between species shows high degree of identity, which suggests that also the cellular function is highly conserved in evolution (Hanks et al. 1992, Schaller et al. 1992). FAK is widely expressed in most cell lines and tissues (Schaller & Parsons 1994, Hanks & Polte 1997). Another tyrosine kinase known as RAFTK, Pyk2 or CAKβ, is a newly identified protein kinase having high structural similarity with FAK, and therefore these proteins are assumed to comprise a new protein tyrosine kinase family (Lev et al. 1995, Sasaki et al. 1995). Certain cells express an alternatively spliced form of FAK, FRNK (for FAK related non-kinase) which is identical in sequence to the C-terminal third of FAK, hence lacking the kinase domain, and displays a restricted tissue distribution (Hanks & Polte 1997).

![Fig. 2. Schematic structure of focal adhesion kinase. Possible interaction sites with other molecules important in integrin signalling are also shown. FAT; focal adhesion targeting, P1 and P2; proline-rich regions 1 and 2.](image)

The mechanisms for FAK activation by integrins are still poorly understood. Integrin β cytoplasmic domains are known to bind the N-terminal domain of FAK in vitro
Schaller et al. 1995), however, the physical linkage between FAK and the integrin cytoplasmic domain is neither necessary nor sufficient for FAK phosphorylation (Tahiliani et al. 1997). A number of studies demonstrate that FAK activation by integrins is tightly coupled to the assembly of focal adhesions, formation of stress fibres and to the integrity of the actin cytoskeleton. FAK activity is enhanced not only by integrin ligation, but also by several soluble agonists, cytokines and growth factors, which affect the cytoskeleton (Zachary & Rozengurt 1992). Tyrosine phosphorylation and activation of FAK in response to integrins, lysophosphatidic acid, bombesin and platelet-derived growth factor (PDGF) can be blocked by treatment of cells with cytochalasin D that selectively disrupts F-actin filaments (Guan & Chen 1996). Recently, Src kinase was shown to have an important role in enhancing FAK activation following the initial FAK tyrosine autophosphorylation (Schaller et al. 1994, Calalb et al. 1995, Brown & Cooper 1996). Src kinase binds through its Src-homology 2 (SH2) domain (see below) to autophosphorylated Tyr-397 in FAK leading to further phosphorylation and full activation of FAK (Cobb et al. 1994, Schaller et al. 1994, Schlaepfer et al. 1994). As a result of its tyrosine phosphorylation FAK can complex with an adapter protein Grb2. Grb2 is an adaptor protein containing a single SH2 and two SH3 domains (see below). Grb2 binds to the GDP/GTP exchange protein Sos which in turn activates Ras and the Erk kinase cascade of MAPKs (see earlier) (Downward 1994). Therefore Ras/Erk activation by integrin stimulation (Chen et al. 1994, Schlaepfer et al. 1994, Morino et al. 1995, Schlaepfer & Hunter 1997, Schlaepfer & Hunter 1998) could be mediated by FAK-Grb2-Sos association.

Many proteins that co-localise with FAK in focal adhesions and serve as its potential effectors are so-called docking or adapter proteins. They are cytoplasmic signalling proteins that do not carry any enzymatic activity, but mediate protein-protein interactions (Lo et al. 1994, Turner 1994). These interactions are mediated through specific, highly conserved protein modules, several of which have been described during the last few years. Src-homology-2 (SH2) and Src-homology-3 (SH3) domains (Pawson 1995), which contain related sequences of 50-100 amino acids, were the first domains identified that mediate protein-protein interactions. SH2 and SH3 domains each recognize short peptide motifs bearing phosphotyrosine in the case of SH2, or one or more proline residues in the case of SH3. The sequence context of the phosphotyrosine or proline residues dictate which SH2 or SH3 domains (and therefore which signalling proteins) are bound. Several other domains are also known, some of which are regulated by post-translational modifications such as by tyrosine phosphorylation, while others appear to be constitutive in nature. Docking proteins capable of mediating protein-protein interactions have been shown to control the location of the signalling proteins within the cell, the enzymatic activity of other proteins and the cytoskeletal interactions and organisation inside the cells (Pawson 1995). Therefore, they are considered as important mediators and regulators of cell signalling.

Docking proteins tensin, paxillin and p130Cas, that co-localise with FAK in focal adhesions (Schaller et al. 1992, Hanks et al. 1992, Turner & Miller 1994, Petch et al. 1995), have been identified as tyrosine-phosphorylated proteins during integrin-mediated cell adhesion (Burrige et al. 1988, Bockholt & Burrige 1993, Nojima et al. 1995, Petch et al. 1995, Vuori & Ruoslahti 1995). The docking protein p130Cas, which is a putative
direct FAK effector and an emerging central molecule in integrin signalling, is described in detail below.

2.2. Cas, a docking protein implicated in multiple signalling pathways

2.2.1. Structure of Cas

p130Cas (Cas, Crk-Associated Substrate) is a 130-kDa protein originally identified as a highly tyrosine-phosphorylated protein in p47\textsuperscript{v-Crk} (v-Crk) (Matsuda et al. 1990, Birge et al. 1992) and p60\textsuperscript{v-Src} (v-Src) transformed cells (Kanner et al. 1990, Kanner et al. 1991). Molecular cloning (Sakai et al. 1994a) showed Cas contained several domains capable of mediating protein-protein interactions, but has no enzymatic activity (see Fig. 3.). Cas contains an N-terminal SH3 domain followed by a proline-rich region, while another proline-rich region is located in the C-terminal part of the protein. Between the tyrosines 377 and 414 is a so-called substrate domain with a cluster of tyrosines having similar adjacent sequences at variable intervals. There are a total of 15 potential phosphotyrosine sites that could be recognized by SH2 domain-containing proteins. Nine of these are YDV/TP sequences that conform to the binding site of Crk SH2 domain (Songyang et al. 1993). Towards the C-terminus from the substrate domain is a serine-rich region and a Src SH2/SH3 binding domain (Nakamoto et al. 1996). A conserved YDYVHL motif in this Src binding region serves as a potential binding site also for the protein tyrosine phosphatase SHPTP2 SH2 domain (Minegishi et al. 1996). The structure of Cas suggests it may play a role in assembling multiprotein complexes in focal adhesion sites (Nakamoto et al. 1997).

Fig. 3. Schematic structure of Cas. Interaction sites with other putative integrin signalling proteins are shown. P1 and P2; proline-rich regions 1 and 2, Y-P; potential phosphotyrosine residues. At least 15 tyrosine phosphorylation sites are likely to exist in the substrate domain of Cas.

2.2.2. New members of the Cas protein family
Two recently cloned molecules, HEF1 (human enhancer of filamentation1)/Cas-L (Law et al. 1996, Minegishi et al. 1996) and Efs (embryonal Fyn-associated substrate)/Sin (Src-interacting or signal-interacting protein) (Ishino et al. 1995, Alexandropoulos & Baltimore 1996) have high structural similarity with Cas, and therefore these proteins are assumed to comprise a new family of docking proteins. Efs/Sin is a 83 kDa protein and HEF1/Cas-L is a 105 kDa protein. Both show around a 60% similarity with Cas, the overall similarity being highest in the SH3 domain and the C-terminal region, while the substrate domain is more divergent. These proteins contain an N-terminal SH3 domain, multiple YXXP motifs, binding sites for the Src-family proteins and a conserved C-terminus. HEF1/Cas-L contains 13, and Efs/Sin eight repeated YXXP motifs in the substrate domain. Efs/Sin lacks the second proline-rich region towards the C-terminus of the molecule, which is found in the two other Cas family members. Distribution of the Cas-family members varies; while Cas is expressed ubiquitously, HEF1/Cas-L is highly expressed in kidney, lung, placenta and lymphocytes (Law et al. 1996) and Efs/Sin is highly expressed in placenta and brain (Ishino et al. 1995). Since the structure of the Cas family members is conserved, it is not surprising that the interactions of these proteins with kinases and other signalling proteins are similar.

2.2.3. Tyrosine phosphorylation of Cas

Cas is widely expressed in all tissues in high concentrations, the highest concentrations being found in testis, intestine and lung. Cas phosphotyrosine content is low in quiescent cells, but Cas becomes highly phosphorylated in cells transformed by v-Crk and v-Src. Tyrosine phosphorylation levels of Cas correlate well with the transforming phenotype of these cells suggesting a potential role for Cas in mediating cellular transformation by these oncproteins (Matsuda et al. 1991, Matsuda et al. 1993). Recently, Cas was shown to become highly phosphorylated in cells transformed by the oncogenic Abl protein (Salgia et al. 1996). Cas has been reported to become tyrosine-phosphorylated in response to a number of different physiological stimuli, many of which affect the formation of focal adhesions and assembly of the actin cytoskeleton. Among these stimuli are integrin mediated cell adhesion (Nojima et al. 1995, Petch et al. 1995, Vuori & Ruoslahti 1995), ligation of B-cell receptor and interleukin-8 receptor (Schraw & Richmond 1995, Ingham et al. 1996) and stimulation of cells with nerve growth factor (NGF) (Ribon & Saltiel 1996), platelet derived growth factor (PDGF) (Rankin & Rozengurt 1994, Casamassima & Rozengurt 1997), bombesin and other mitogenic neuropeptides, phorbol esters and bioactive lipids (Rozengurt 1995, Casamassima & Rozengurt 1997).

2.2.4. Regulation of tyrosine phosphorylation of Cas
Cas-FAK interaction is thought to be important in the regulation of Cas phosphorylation. FAK has been shown to phosphorylate Cas in vitro (Schlaepfer et al. 1997, Vuori et al. 1996), but there is also evidence that FAK could phosphorylate Cas in vivo; expression of the constitutively active form of FAK results in constitutive tyrosine phosphorylation of Cas (Vuori et al. 1996). FAK or the FAK homologue RAFTK/Pyk2/CAKβ was recently shown to initiate the tyrosine phosphorylation of Cas by directly phosphorylating the Src SH2 domain binding site in Cas (Tachibana et al. 1997), resulting in Cas-Src interaction and further phosphorylation of Cas. FAK could also recruit Src family kinases via autophosphorylation of Tyr-397, thereby serving as a link between Cas and Src (Schaller et al. 1995, Vuori et al. 1996, Richardson et al. 1997). New insights into the complex regulation of Cas phosphorylation have been obtained by the recent findings of tyrosine phosphatase interactions with Cas. Overexpression of the tyrosine phosphatase PTP1B in v-Crk transformed cells results in dephosphorylation of Cas (Liu et al. 1996). Cas has also been identified as a highly specific substrate in vitro and in vivo for yet another phosphatase, PTP-PEST (Garton et al. 1996). However, the molecular events leading to Cas dephosphorylation are still poorly understood.

2.2.5. Protein-protein interactions by Cas

The phosphotyrosine content of Cas has been found to correlate with its binding to other signalling molecules. Thus, in vivo association of Cas with Src in v-src-transformed cells (Reynolds et al. 1989, Kanner et al. 1991) and with Crk in v-Crk-transformed cells (Sakai et al. 1994b) has been reported. Cas interaction with the Crk SH2 domain was shown to be adhesion-dependent in non-transformed fibroblasts (Hamasaki et al. 1996, Vuori et al. 1996). Crk is a member of an adapter protein family that consists mostly of SH2 and SH3 domains and carries no enzymatic activity. Crk was originally identified as its viral form encoded by avian sarcoma virus CT10, in which viral gag sequences are fused to cellular Crk sequences coding primarily the SH2 and one of the two SH3 domains. Two cellular homologs c-Crk-I and c-Crk-II are produced from the same gene by alternative splicing, whereas another cellular form, c-Crk-L, is encoded by a distinct gene (Mayer et al. 1988). Each Crk protein contains one SH2 domain, c-Crk-I carries only one SH3 domain, while c-Crk-II and c-Crk-L have two SH3 domains (Matsuda et al. 1992). Two guanine nucleotide exchange factors for small GTPases, C3G and Sos, which interact with Crk's SH3 domain, have been detected in the Cas complex (Vuori et al. 1996). These interactions represent a potential route from cell adhesion to the Ras pathway. In vitro binding studies with GST-immobilised SH2 domains of PI 3-kinase, phospholipase C-γ and adaptor proteins Grb2 and Nck, showed that these proteins can interact with Cas from lysates of adherent but not of suspended fibroblasts (Vuori et al. 1996, Schlaepfer et al. 1997). In addition, in vitro binding of the SH2 domain of tensin with Cas has been demonstrated (Lo et al. 1994). However, it remains to be determined whether these interactions take place in intact cells. Recently, Cas was reported to associate with 14-3-3 proteins through phosphorylated serine residues (Garcia-Guzman et al. 1999), which implicates that also serine-phosphorylation events may regulate Cas interactions with other proteins. Phosphorylation-independent interactions of Cas SH3 domain with
proline-rich regions of FAK (Polte & Hanks 1995), guanine exchange factor C3G (Kirsch et al. 1998) and protein tyrosine phosphatases PTP-PEST and PTP1B were recently demonstrated (Liu et al. 1996, Garton et al. 1997).

2.3. Functional role of focal adhesion proteins in integrin signalling

Recent evidence indicates a role for focal adhesion proteins in controlling cell spreading and migration downstream of integrins. For example expression of FAK in vivo is increased in rapidly migrating and invasive cells (Gates et al. 1994, Akasaka et al. 1995, Owens et al. 1995). Lack of FAK function or inhibition caused by expressing dominant-negative acting FRNK in cells or by using FAK-negative embryo derived cells results in impaired spreading and reduced rates of migration (Ilic et al. 1995, Richardson & Parsons 1996). Overexpression of wild-type FAK increases migration of CHO cells, which requires FAK-Src complex formation (Cary et al. 1996). There is evidence that Cas may act as a downstream mediator of FAK promoted cell migration. First, a mutant form of FAK that does not bind Cas fails to promote cell migration, and secondly, co-expression of wild-type Cas with FAK in CHO cells further enhances the migration promoted by FAK alone (Cary et al. 1998). Evidence points to the role of Cas-Crk signalling complex formation in integrin-mediated haptotactic and cytokine-induced cell migration (Klemke et al. 1998). Expression of wild-type Cas or Crk alone is sufficient to promote cell migration, whereas mutants of these proteins that were unable to interact with each other failed to do so. Crk SH2 domain binding to the Cas substrate domain was required for the induction of cell migration, but an intact Crk N-terminal SH3 domain was also necessary. Hence, cell migration induced by EGF and insulin, known regulators of cell motility, required functional Cas and Crk, which were driven to the membrane ruffles in migratory cells (Klemke et al. 1998).

Both cell attachment to the ECM by integrins and mitogenic stimulation by growth factors are essential for most types of normal cells to proliferate and to survive (Frisch & Ruoslahti 1997). Apoptosis of suspended cells can be prevented by elevating tyrosine phosphorylation (Meredith et al. 1993); which together with additional evidence suggests a role for adhesion induced kinases in this process. First, epithelial cells can be rescued from detachment induced apoptosis (anoikis) by transfection with a constitutively active form of FAK, and the FAK-Src complex formation correlates with cell survival (Frisch et al. 1996). Second, inactivation of FAK with antibodies induces apoptosis in fibroblasts (Hungerford et al. 1996), and third, the ECM survival signals transduced by FAK inhibit tumour suppressor p53 mediated apoptosis (Ilic et al. 1998). Interestingly, many invasive tumours display elevated expression of FAK, which may contribute their anchorage-independent growth (Owens et al. 1995). Expression of an activated form of FAK induces anchorage-independent but serum-dependent growth of epithelial cells and renders these cells tumourigenic (Frisch et al. 1996). Thus, the cell survival signalling of FAK may be an important factor also in FAK induced tumourigenesis.
2.4. Cbl, a novel tyrosine-phosphorylated protein in integrin signalling

In many cell systems FAK, paxillin and Cas are among the best characterised tyrosine-phosphorylated proteins in integrin signalling. The present studies demonstrate that in myeloid cells the main tyrosine-phosphorylated protein involved in integrin signalling was not any of the known integrin signalling proteins, but p120\textsuperscript{Cbl}, a protein previously implicated in growth factor signalling in hematopoietic cells.

2.4.1. Structure of Cbl

p120\textsuperscript{Cbl} (Cbl) was first identified as the cellular homologue of the transforming v-Cbl protein of Cas NS-1 murine retrovirus that induces pre-B cell lymphomas and myeloid tumours in mice (Langdon et al. 1989a). Cloning of cellular Cbl (c-Cbl) revealed that the genome of v-Cbl represents a fusion of the first 355 N-terminal residues of the full-length (913 amino acids) c-Cbl protein to viral gag sequences (Blake et al. 1991). The full-length c-Cbl is most abundantly expressed in thymus and testis tissues and in hematopoietic cells, and carries no intrinsic enzymatic activity (Langdon et al. 1989b). Cbl contains an N-terminal region with a potential nuclear localisation sequence, which apparently is not used. Neither c-Cbl nor oncogenic forms of Cbl devoid of viral gag sequences localise to the nucleus, but are exclusively cytoplasmic. In the C-terminal half of c-Cbl lie several potential tyrosine phosphorylation sites, proline-rich sequences and a putative leucine zipper, a motif known to promote homo- and heterodimerization of other proteins (Blake et al. 1993). In between the N-terminal and C-terminal halves of Cbl there is also a RING finger domain, a motif which is thought to mediate protein-protein or protein-DNA interactions (Lovering et al. 1993, Saurin et al. 1996). Recently a new N-terminal domain which binds phosphotyrosines (PTB for phosphotyrosine binding) was identified in Cbl (Bowtell & Langdon 1995, Lupher et al. 1996). Thus, c-Cbl is well suited in mediating protein-protein interactions (see Fig. 4.). c-Cbl does not show any transforming potential even when overexpressed (Blake et al. 1993, Andoniou et al. 1994). Recently, a Cbl-related gene product, Cbl-b, was isolated from human breast cancer cells (Keane et al. 1995). Cbl-b is expressed in a variety of normal tissues and cell lines (Keane et al. 1995). The primary structure of c-Cbl and Cbl-b gene products shows high similarity, suggesting a similar biological function for both proteins in mammalian cells. A cbl homologue in the nematode Caenorhabditis elegans, SLI-1 (suppressor of lineage defect-1), was found by genetic studies of suppressors of vulval development defects arising from mutations of the LET-23 receptor, the homolog of the mammalian EGF receptor (Jongeward et al. 1995, Yoon et al. 1995) Sli-1 shows a 43% similarity to human Cbl, the similarity being highest in the N-terminus and the ring finger domain. A Drosophila homolog of Cbl, D-Cbl, was recently reported (Hime et al. 1997, Meisner et al. 1997); it also shows high homology with Cbl in the N-terminus but lacks the proline-rich regions. The function of the wild-type c-Cbl is not known while the oncogeneity of its various mutant forms (see below) is well documented.
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2.4.2. Oncogenic forms of Cbl

The transforming capacity of c-Cbl can be achieved by several different mutations. A mutant form of Cbl cloned from the mouse pre-B cell lymphoma cell line 70Z/3 was shown to carry a deletion of amino acids 366 to 382. This 17 amino acid deletion immediately N-terminal to the Ring finger domain and C-terminal to the v-Cbl coding region was sufficient to convert c-Cbl into an acutely transforming protein, demonstrated by expressing 70Z/Cbl in NIH 3T3 fibroblasts (Andoniou et al. 1994). Single amino acid deletions of Tyr-368 or Tyr-371 within this 17 amino acid stretch are also sufficient to induce the transforming potential of Cbl. Transfection of NIH 3T3 fibroblasts with retroviral vectors expressing these mutated forms of Cbl resulted in high efficiency colony formation in soft agarose assay and in an ability to promote tumor growth in nude mice (Andoniou et al. 1994). The 17 amino acid deletion and the Tyr-368 and Tyr-371 deletions in Cbl induce a marked elevation in tyrosine phosphorylation status of Cbl (Andoniou et al. 1994). Normally only a small fraction of Cbl is tyrosine-phosphorylated, whereas up to 100% of the mutant proteins appear to have phosphorylated tyrosine residues. These mutations result in the constitutive tyrosine phosphorylation of Cbl, which correlates with an ability to promote tumor growth. This suggests that deregulated tyrosine phosphorylation of Cbl can lead to tumourigenesis.
2.4.3. Tyrosine phosphorylation and tyrosine kinase interactions of Cbl

Cbl is believed to function in intracellular signalling pathways based on its structure which is suitable for protein complex formation and on the observation that Cbl becomes tyrosine-phosphorylated in different cell types in response to stimulation through a variety of cell-surface receptors. Tyrosine phosphorylation of c-Cbl has been reported to occur in response to number of stimuli, including ligand induced stimulation of T- and B-cell antigen receptors, cytokine receptors such as the granulocyte-macrophage colony-stimulating factor, interleukin-2, interleukin-3, interleukin-4 and erythropoietin receptors, colony-stimulating factor-1 (CSF-1), thrombopoietin TPO/c-mpl and interferon-α receptor. Activation of lymphocyte co-stimulatory receptors CD38 and CD28 and immune complex receptors FcγR, FcεRI induce the same response (for a review, see Miyake et al. 1997). Stimulation of cell surface receptors that do not possess intrinsic tyrosine kinase activity results in activation of the members of non-receptor tyrosine kinase families, such as Src and Syk/Zap70. Interaction of Cbl with these activated kinases is thought to contribute to Cbl tyrosine phosphorylation. A current model for Cbl tyrosine phosphorylation in T- and B-cells considers Syk/Zap-70 the principal initiator and phosphorylator of Cbl, whereas the Src family kinases contribute indirectly to this process by the activation of Syk/Zap-70 (for a review, see Liu & Altman 1998). Cbl is constitutively hyperphosphorylated in cells expressing oncogenically activated forms of Src and Lck kinases (Miyake et al. 1997, Liu & Altman 1998). Cbl also becomes highly tyrosine-phosphorylated and associates \textit{in vivo} with the abl SH2 domain in cells transformed with the v-abl and bcr-abl oncogenes (Andoniou et al. 1994). Constitutive, oncogenic activation of abl is a result of retroviral alterations, or of a chromosomal translocation resulting in formation of a bcr-abl fusion protein as seen in Philadelphia chromosome positive acute lymphoblastoid and chronic myelogenous leukemias (Shtivelman et al. 1985, Wang 1993). Thus, tyrosine phosphorylation of Cbl is thought to play a significant role in abl-mediated tumourigenesis.

Tyrosine phosphorylation of Cbl also occurs in response to stimulation of cells by several growth factors. Activation of receptor tyrosine kinases by stimulation with EGF, PDGF, fibroblast growth factor and NGF, as well as stem cell factor results in tyrosine phosphorylation of Cbl. Cbl associates via its PTB domain with EGF (Bowtell & Langdon 1995, Galisteo et al. 1995, Meisner & Czech 1995, Fukazawa et al. 1996) and PDGFα growth factor receptors (Bonita et al. 1997) after receptor ligand binding. The binding of Cbl to the EGF receptor correlates with tyrosine autophosphorylation of the receptor. EGF receptor kinase activity was shown to be essential for Cbl tyrosine phosphorylation, although it is not known if the receptor itself or another kinase activated by the EGF receptor is responsible for the phosphorylation (Galisteo et al. 1995, Langdon 1995). Recently, oncogenic constitutively tyrosine-phosphorylated forms of Cbl were found to show enhanced binding to the EGF and PDGFα receptors and to upregulate the signalling of these receptors (Bonita et al. 1997, Thien & Langdon 1997).

Taken together, Cbl seems to be a substrate for different PTK families. It is notable that Src family kinases are known to be involved in signalling through many receptors whose stimulation induces Cbl phosphorylation, and these kinases may contribute to Cbl tyrosine phosphorylation in many receptor systems.
2.4.4. Interactions of Cbl with other signalling proteins

In addition to interacting with several tyrosine kinases, Cbl associates with a number of adaptor proteins implicated in tyrosine kinase signal transduction. Interaction between Cbl and the Crk family of adapter proteins in an activation-dependent way has been reported by several investigators (de Jong et al. 1995, Sawasdikosol et al. 1995, Ribon et al. 1996). This interaction is mediated by the SH2 domain of Crk and phosphotyrosine residues in Cbl, most likely Y774 and Y700 in the C-terminal region of Cbl (Andoniou et al. 1996, Reedquist et al. 1996). Cbl-Crk interaction may couple proximal PTKs to the activation of small GTP-binding proteins C3G and Sos (Tanaka et al. 1994). Cbl has also been shown to associate directly through its proline-rich region with the SH3 domain of Nck (Rivero-Lezcano et al. 1994) and with the N-terminal SH3 domain of Grb2 (Donovan et al. 1994, Fukazawa et al. 1995, Meisner et al. 1995, Odai et al. 1995). Since Grb2 interacts with Sos via the same SH3 domain as with Cbl, Cbl-Grb2 interaction cannot present another route to the Ras signalling pathway. Vav (Collins et al. 1997), a putative exchange factor for small GTP-binding-proteins Rac and Rho expressed only in hematopoietic cells, was recently reported to co-immunoprecipitate with Cbl in T-cells in an activation-dependent manner (Marengere et al. 1997). This interaction was suggested to occur through the SH2-domain of Vav binding to the phosphotyrosine Y700 in Cbl (Marengere et al. 1997). Cbl-b overexpression inhibits Vav-mediated JNK activation suggesting a functional role for Cbl-Vav association (Bustelo et al. 1997). Studies in several cell types have demonstrated that stimulation through tyrosine kinase-coupled receptors induces a prominent association of tyrosine-phosphorylated Cbl with PI 3-kinase (Liu & Altman 1998), the products of which are thought to mediate signalling from activated cell surface receptors to a number of signalling events (Toker & Cantley 1997). Interaction between Cbl and PI 3-kinase is mediated by the SH2 domain of the PI 3-kinase regulatory subunit (p85) (Fukazawa et al. 1995, Kim et al. 1995) and phosphotyrosine residues, probably Y731 in Cbl (Liu et al. 1997).

2.4.5. Biochemical and biological functions of Cbl

Several studies provide evidence for the role of c-Cbl as a regulator of tyrosine kinases. Genetic studies in C. elegans revealed that SLI-1, a homolog of mammalian Cbl acts as a negative regulator of EGF signalling (Jongeward et al. 1995, Sternberg et al. 1995, Yoon et al. 1995). Later, a Drosophila homologue of Cbl, D-Cbl, was reported to bind EGF receptor and to function as a negative regulator of the receptor kinase-mediated R7 photoreceptor cell development (Hime et al. 1997, Meisner et al. 1997). In mammalian systems, Cbl overexpression in fibroblasts was shown to reduce the autophosphorylation and signalling of the EGF receptor, and cell transfection with anti-sense Cbl constructs was shown to enhance EGF receptor phosphorylation and signalling through the JAK-STAT pathway (Ueno et al. 1997). Further, Cbl overexpression negatively regulated tyrosine phosphorylation and kinase activity of Syk in FcεRI-stimulated mast cells (Ota & Samelson 1997). As discussed earlier, oncogenic mutants of Cbl bind to EGF and PDGFα receptors and upregulate their signalling (Bonita et al. 1997, Thien & Langdon 1997).
further pointing to a role of cellular Cbl as a negative regulator of receptor tyrosine kinases. One possible mechanism for this negative regulation was suggested when Cbl overexpression was found to result in enhanced ubiquitination and degradation of the PDGFα receptor (Miyake et al. 1998). Cbl was also shown to ubiquitinate the EGF receptor, resulting in degradation of the receptor in the lysosomal compartment of the cell (Levkowitz et al. 1998). Already earlier, CSF-1 receptor stimulation has been found to induce Cbl ubiquitination and membrane translocation possibly through a ubiquitin-binding domain in the C-terminus of Cbl (Hofmann & Bucher 1996, Wang et al. 1996).

Though it seems that most of the data reported to date supports the view of Cbl as a negative regulator of tyrosine kinase signalling, there are also reports supporting the opposite view. Tanaka et al. suggested that Cbl is a positive regulator acting downstream of Src in the signalling pathway leading to bone resorption (Tanaka et al. 1997), whereas Ueno and coworkers reported c-Cbl to mediate positive signals in IL-4 induced mitogenic and survival signalling through PI 3-kinase (Ueno et al. 1998).
3. Outlines of the present research

Cellular sites known as focal adhesions have been found to be key regions for integrin-mediated signalling events in many adherent cell types. Tyrosine phosphorylation events in focal adhesions are considered to play an important role in the regulation of integrin intracellular signalling; the main focus of the present research was to study these events. Notably, the docking protein p130Cas (Cas) resides in these sites and has been found to become tyrosine-phosphorylated in response to integrin-mediated cell adhesion and other cellular stimuli. As a result of tyrosine phosphorylation, Cas engages in multiple protein-protein interactions, such as that with the adapter protein Crk, and may activate downstream signalling pathways. At the time this work was initiated, relatively little was known about the effects of growth factor stimulation on Cas or about the biochemical signalling pathways which involve Cas. These issues were examined in the first part of this study. While integrin-mediated signalling events are relatively well characterised in adherent cells such as fibroblasts, little is known about these events in hematopoietic cells. In the latter part of this study, the focus was on the integrin-induced signalling events in hematopoietic cells of the myeloid lineage. The docking protein p120Cbl (Cbl) was identified as one of the main tyrosine-phosphorylated proteins in myeloid cells, and additional studies were outlined to further characterise the role of Cbl in integrin signalling.

1. to study the effects of EGF on Cas phosphorylation and signalling.
2. to study the functional significance of the Cas-Crk complex signalling in JNK MAP kinase activation.
3. to identify and characterise the integrin-induced tyrosine phosphorylation events in hematopoietic cells of the myeloid lineage. The docking protein p120Cbl (Cbl) was identified as one of the main tyrosine-phosphorylated proteins in myeloid cells, and additional studies were outlined to further characterise the role of Cbl in integrin signalling.
4. to study the mechanisms of transformation by oncogenic forms of Cbl with an emphasis on adhesion and growth factor-dependent events.
4. Materials and methods

4.1. Cell culture and cell adhesion experiments (I, II, III, IV)

Various cell lines were used in the experiments as indicated in the results. Mouse macrophage cell lines IC-21, P388D1 and RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI (Mediatech, Herndon, VA, USA) supplemented with 10% fetal calf serum (FCS) (Tissue Culture Biologicals, Tulare, CA, USA), 50 units/ml penicillin and 50 µg/ml streptomycin (Irvine Scientific, Santa Ana, CA, USA). Rat-1 cells expressing human EGF receptor (RatER) (Moran et al. 1990), epidermoid carcinoma A431 cells (Peppelenbosch et al. 1993), wild-type Rat-1 cells, NIH 3T3 fibroblast cell lines expressing wild-type (c-Cbl) and transforming mutants of Cbl (70Z/Cbl, Y368del and Y371del) (Andoniou et al. 1994), HeLa and COS-7 cells were grown in Dulbecco’s modified Eagles medium (DMEM) (Mediatech) supplemented with 10% FCS, 50 units/ml penicillin and 50 µg/ml streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere. For the experiments, cells were grown to 90% confluency as monolayers. For cell adhesion experiments, macrophage cell lines were scraped off the dish with a cell scraper and the other cell lines were detached with trypsin-EDTA treatment followed by washing with soybean trypsin inhibitor. The cells were collected by centrifugation, washed twice with the appropriate medium containing 0.5% bovine serum albumin (BSA), and cell suspensions were incubated in a medium with 0.5% BSA at 37°C for 20 minutes on a rotator. Cells referred to as adherent in the “Results” were then plated onto dishes coated with various substrates and incubated at 37°C for the times indicated. Cells referred to as suspended were held in suspension for an additional 20 minutes. HeLa and COS-7 cells were detached by trypsinization 48 hours after transfection and either lysed immediately or re-attached in fibronectin coated dishes for 15 minutes in 37°C. The various substrates, fibronectin (20 µg/ml), vitronectin (20 µg/ml), affinity-purified anti-α5β1 antibody (10 µg/ml), affinity-purified anti-αvβ3 antibody (10 µg/ml), monoclonal anti-major histocompatibility complex (anti-MHC) antibody (10 µg/ml) or polylysine (20 µg/ml) used to coat the dishes were incubated on the dishes overnight, and blocked with 0.5% BSA for 1 hour prior to plating the cells.
4.2. Cell transfections and plasmids (II)

HeLa and COS-7 cells were used in transfection studies. Transient transfections were carried out using either the lipofectamine or the calcium phosphate method. Lipofectamine was used according to the manufacturer's instructions (Gibco-BRL, Gaithersburg, MD, USA). For the transfections with the calcium phosphate method, cells were plated on 35 mm cell dishes at a density of $3 \times 10^5$ cells/well. Purified DNA (2.4 µg) were used for transfection on each well for 24 hours. The cells were rinsed with phosphate-buffered saline (PBS) and re-fed with serum-free media for 18-24 hours before lysis.

The Crk-constructs used in this study have been described in (Matsuda et al. 1992, Tanaka et al. 1993). pCAGGS-expression vectors for DOCK180, C3G and Sos have been described in (Matsuda et al. 1994, Tanaka et al. 1994, Hasegawa et al. 1996). The expression vector for dominant-negative Rap-1, pCAGGS-(Myc)Rap1N17, was constructed in the laboratory of Michiyuki Matsuda, Tokyo, Japan. The pCAGGS-constructs contain a myc-epitope in the N-terminus of the protein produced. The wild-type Cas-expression construct has been described in (Sakai et al. 1994b). The expression vector for CasΔSD lacking the substrate domain (amino acids 213-514) so that it lacks the ability to bind the Crk SH2 domain (Sakai et al. 1994b), was constructed as in (Nakamoto et al. 1996). pSRα3-(HA)JNK1, pCMV5-(M2)JNK1, pSRα3-Rac1N17, and pSG-v-Src have been described in (Minden et al. 1994, Cavigelli et al. 1995, Minden et al. 1995). pEBB-constructs for the wild-type Grb2 and the SH2-mutant of Grb2 (R86K) are described (Tanaka et al. 1995). The pEGFP-N1-vector coding for the green fluorescent protein is from Clontech (Palo Alto, CA, USA).

4.3. Cell stimulants and inhibitors (I, II, III)

For the EGF stimulation, cells were serum-starved for 24 or 36 hours prior to the experiments. EGF (Calbiochem, San Diego, CA, USA) was added to the culture media in various concentrations without detaching the cells and the cells were maintained at 37°C for the times indicated. In order to inhibit PI 3-kinase activity, LY294002 (Calbiochem), which has been identified as a specific inhibitor of PI 3-kinase (Vlahos et al. 1994) was added to the media at several concentrations (5, 10 or 20 µM) for two hours prior the experiments (with or without subsequent EGF stimulation, as indicated in the text). To verify the results obtained with LY294002, the structurally and functionally unrelated PI 3-kinase inhibitor wortmannin (Calbiochem) was used in some experiments at concentrations of 20 nM, 50 nM and 100 nM (see Nakanishi et al. 1992, Yano et al. 1993). In order to disrupt the actin cytoskeleton organisation in the experiments indicated in the text, preincubations were done with various concentrations (0.05, 0.25, 1.0, 2.0 µM) of Cytochalasin D (Bockholt & Burridge 1993) for the times indicated (10 minutes or 2 hours). After Cytochalasin D treatment cells were either stimulated by CSF-1 or EGF or left unstimulated. CSF-1 stimulation of the cells (see Wang et al. 1996) was done with a concentration of 25 nM for 1, 3, 5 or 10 minutes. CSF-1 from L-cell conditioned
medium (Celada & Maki 1992) was obtained from Dr. Richard Maki (The Burnham Institute, La Jolla, CA, USA).

4.4. Preparations of cell lysates (I, II, III, IV)

Cells were washed with ice-cold PBS and lysed on ice for 15 minutes in modified radioimmune precipitation assay (RIPA) buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na3VO4, 1 mM EGTA, 0.1 U/ml aprotinin, 10 µg/ml leupeptin and 4 µg/ml pepstatin A), Nonidet P-40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and phosphatase and protease inhibitors as above) was used in experiments where in vitro kinase assays were carried out, and Nonidet P-40/Triton X-100 buffer (20 mM Tris, pH 7.5, 145 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1% Triton X-100, 5 mM EDTA and phosphatase and protease inhibitors) was used for PI 3-kinase assays. For JNK kinase assays, cells were lysed in ice cold kinase lysis buffer (50 mM Heps, pH 7.6, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 100 µM Na3VO4, 0.5% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1mM phenylmethylsulphonylfluoride (PMSF)) for 15 minutes. Lysates were cleared by centrifugation for 15 minutes at 12 000 rpm at +4°C. The supernatants were normalised for the protein content as determined by the DC protein assay (Bio-Rad, Hercules, CA, USA) and used for the experiments.

4.5. Immunoprecipitations and immunoblot analysis (I, II, III, IV)

Antibodies used in this study were polyclonal anti-Cas antibody (C-20), polyclonal rabbit anti-Chi antibody, anti-hemagglutinin (HA, clone Y-11) (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) and polyclonal anti-Crk antibody (from Dr. Michiyuki Matsuda, National Institute of Health, Tokyo, Japan). Monoclonal anti-c-CrkII-, anti-Cas-, anti-Grb2-, anti-PI 3-kinase-, anti-FAK-, anti-paxillin-, anti-p120Ras-GAP-, anti-phosphotyrosine- (py20), and horseradish peroxidase (HRPO)-conjugated py20 antibodies were from Transduction Laboratories (Lexington, KY, USA). Monoclonal anti-vinculin antibody was from Sigma (St. Louis, MO, USA). Anti-Myc antibody was from Calbiochem and anti-Flag (M2) from Sigma. Anti-mouse MHC class I H-2 monoclonal antibody (American Type Culture Collection), polyclonal rabbit anti-α5β1 and anti-αvβ3 antibodies, which recognize the mouse integrins (Vuori & Ruoslahti 1995), were from Dr. Erkki Ruoslahti (The Burnham Institute). Rat anti-mouse β1 integrin antibody was from Pharmingen (San Diego, CA, USA). Monoclonal anti-Src antibody 327 was obtained from Dr. Joan Brugge (Harvard Medical School, Boston, MA, USA).

Antibodies were added to lysates containing equal amounts of protein, and samples were incubated on ice for 1 hour. To precipitate antibody-antigen complexes, 20 µl of a 50% suspension of Gammabind-Sepharose or protein-A Sepharose (Amersham-Pharmacia, Piscataway, NJ, USA) beads were added to the lysates following rotation at +
4°C for 2 hours. The immunoprecipitates were collected by centrifuging and washed three times in wash buffer (lysis buffer without detergents). Pellets were then boiled in SDS sample buffer and electrophoresed on 4-12% precast SDS-polyacrylamide gel electrophoresis gels (Novex, Encinitas, CA, USA). The separated proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) and the membranes blocked overnight in PBS containing 0.5% BSA. Phosphotyrosine containing proteins were detected by incubation with HRPO-conjugated anti-phosphotyrosine py20 antibody (Transduction laboratories) for 60 minutes at room temperature with rotation followed by serial washing with PBS/1% BSA/0.1% Tween-20. Visualisation of the bands was performed by enhanced chemiluminescence detection (Pierce, Rockford, IL, USA). To detect specific proteins, blots were probed with the corresponding monoclonal or polyclonal antibodies, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG and protein A, respectively. Immunoreactive bands were visualised by enhanced chemiluminescence. Epitope-tagged proteins were immunoprecipitated by specific antibodies against the tag and the blots were probed with the corresponding antibodies.

4.6. SH2- and SH3- binding assays and blotting with GST-fusion proteins (III, IV)

In vitro association experiments were done with glutathione-S-transferase (GST) fusion proteins containing the SH2 domains of Crk, PI 3-kinase (this construct contains the N-terminal SH2 domain of PI 3-kinase (Jhun et al. 1994)), Shc (Sasaoka et al. 1994) and Src, and the SH3 domains of Crk, PI 3-kinase and Src. The GST-SH2 domain of Crk was from Dr. Michiyuki Matsuda (Research Institute, International Medical Center of Japan, Tokyo, Japan), and GST-SrcSH2 from Dr. Hisamaru Hirai (University of Tokyo, Tokyo, Japan). The GST-SH3 domains of Crk, PI 3-kinase and Src were from Dr. Stephen Taylor (Cornell University, Ithaca, NY, USA). The fusion proteins were expressed in Escherichia coli and purified as described in (Vuori et al. 1996). The fusion protein preparations were > 95% pure as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining. Cell lysates containing equal amounts of protein were incubated with 5 μg of GST alone or with the GST-SH2 or GST-SH3 fusion proteins, which had been immobilised on Glutathione-Sepharose beads, for 2 hours rotating at + 4°C. The beads were collected and washed twice with RIPA buffer and twice with Tris-buffered saline, and the bound proteins were released by boiling in sample buffer followed by SDS-PAGE and immunoblot analysis (see above). To blot with GST-fusion proteins, immunoprecipitates from boiled RIPA lysates were resolved on SDS-PAGE and transferred to Immobilon-P transfer membrane. After blocking in PBS/1% BSA, the membrane was incubated at room temperature on a rotator with 5 μg/ml of purified GST-SH2 or GST-SH3 fusion proteins for 1 hour. The blots were washed with PBS/1% BSA/0.1% Tween-20 and probed with a monoclonal anti-GST antibody (Sigma). Following washing, blots were incubated with HRPO-conjugated anti-mouse IgG followed by washes and detection with enhanced chemiluminescence.
4.7. In vitro kinase assays (II, III, IV)

4.7.1. Tyrosine kinase assays (III, IV)

For in vitro tyrosine kinase assays, immunoprecipitations were done from suspended or adherent cells lysed in NP-40 buffer as described above. The immunocomplexes were washed four times in NP-40 buffer, once in kinase assay buffer (50 mM Hepes, pH 7.5, 25 mM MgCl₂, 4 mM MnCl₂, 500 µM Na₃VO₄) and suspended in 30 µl of kinase assay buffer containing 4 µM ATP and 10 µCi of [γ-³²P]ATP (6000 Ci/mmol; DuPont NEN, Boston, MA, USA). After incubation for 15 minutes in 30°C, the phosphoproteins were separated by 4-12% SDS-PAGE and subjected to alkaline hydrolysis in 1 M KOH, after which tyrosine-phosphorylated proteins were visualised by autoradiography. A slightly modified version of the kinase assay buffer and incubation time and temperature was used in some experiments (macrophage cell lines); 25°C for 30 minutes in a kinase assay buffer containing Pipes, pH 7.0, 10 mM MnCl₂, 1mM DTT and 0.25 µCi [γ-³²P] ATP (6000 Ci/mmol, DuPort-NEN). To study whether Src exhibits kinase activity in the Cbl immunocomplexes, reactions were carried out in the presence of 5 µg of acid-denatured enolase, a substrate for Src family kinases (Sigma). The relative amounts of Cbl were determined by immunoblot analysis of equal aliquots of the non-labeled immunoprecipitates with the anti-Cbl antibodies. In some of the experiments, the immunocomplex proteins were released after the kinase assay by boiling in 0.5% SDS, 50 mM Tris, pH 7.5, 5 mM EDTA, 10 mM DTT. The supernatant was then diluted 10-fold in RIPA buffer and immunoprecipitated with the anti-Cbl and anti-Src antibodies; the reprecipitates were analysed by autoradiography after SDS-PAGE. Quantitation was performed with an Ambis radioanalytic imaging system.

4.7.2. JNK kinase assay (II)

Cell lysates containing equal amounts of protein were immunoprecipitated with anti-hemagglutinin antibodies (HA, clone Y-11; Santa Cruz Biotechnology) and protein A-Sepharose for 3 hours at + 4°C (see above). The beads were collected and washed three times with lysis buffer (see above) and once with kinase assay buffer (50 mM Hepes, pH 7.6, 10 mM MgCl₂). Immune complex kinase assays were performed in a final volume of 20 µl containing the beads, 10 µM ATP, 1 µCi [γ-³²P]ATP and 5 µg of GST-c-Jun (1-79) (Stratagene, La Jolla, CA, USA) as a substrate for JNK. After incubation at 30°C for 20 minutes, the samples were heated at 95°C in SDS sample buffer for 5 minutes and proteins were separated by SDS-PAGE gel electrophoresis followed by visualisation by autoradiography. In parallel with the kinase assays, equal aliquots of the unlabelled immunoprecipitates were analysed with the corresponding antibodies to determine the expression levels of the transfected proteins.
4.7.3. PI 3-kinase assay (III, IV)

For PI 3-kinase activity assays, cells were either kept in suspension, or plated on various substrates as described and lysed in Nonidet P-40/Triton X-100 buffer (see above). Where indicated, cells were stimulated with CSF-1 (25 nM) for the indicated times prior to lysis. Immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control nonrelated antibody were carried out as described. PI 3-kinase activity of the immunocomplexes was assayed as described in (Whitman et al. 1988).

4.8. Assay for c-Jun transcriptional activity (II)

To study if JNK activation results in stimulation of c-Jun transcriptional activity, activation of the GAL4-c-Jun (1-223) fusion protein containing the yeast GAL4 DNA binding domain and the c-Jun activation domain was analysed by using luciferase as a reporter gene. The reporter plasmid contained five tandem repeats of GAL4 binding element followed by the sequence of the luciferase gene. HeLa cells were cotransfected with 1 μg of the pFR-Luc reporter plasmid, 50 ng of pFA-c-Jun (1-223) (PathDetect Reporting System, Stratagene) and the plasmids indicated in the text. After transfection, the cells were serum starved for 24 hours and lysed. The expression of luciferase was assayed using the Luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

4.9. Cell growth assays: proliferation, soft agar and thymidine incorporation assays (I, IV)

To study the growth rate and growth factor dependency of cell proliferation, cells were plated on 35 mm wells at 10,000 cells/well in medium containing 10% FCS. After 12 hours, the media were changed to media containing various concentrations of FCS and the cells were re-fed with the respective media every 3 days. At the time points indicated, cells were detached with trypsin-EDTA and counted in a hemocytometer. The exponential doubling time and saturation density were determined using the results obtained.

For anchorage-independent growth assays (soft agar assay), 1,000 cells were suspended in 1.5 ml of 0.3% purified agar in DMEM in the presence of 0.5% or 10% FCS and seeded in 35 mm wells coated with 1.5 ml of 0.5% agar in the respective media. After twenty days the number of colonies of eight cells or more were counted using a microscope.

For thymidine incorporation assays, cells were plated on 96-well dishes at 10,000 cells/well in DMEM containing 10% FCS. After 12 hours the medium was switched to DMEM containing 0.5% FCS and cells were starved for 48 hours. Fresh medium containing 0.5% FCS or 10% FCS was added to the cells 15 hours before a 3-hour pulse-labelling with [3 H]thymidine (1 μCi/well). The amount of incorporated radioactivity was measured by an automatic scintillation counter (Vuori & Ruoslahti 1994).
4.10. Cell fractionation (III)

Hypotonic lysis and fractionation were performed as described in (Hartley & Corvera 1996). Briefly, IC-21 cells adherent either on polylysine or on fibronectin for 40 minutes were incubated on ice in a hypotonic buffer (10 mM Tris, pH 7.5, 0.5 mM MgCl₂ and phosphatase and protease inhibitors as above) for 10 minutes, and then scraped off and homogenised in a glass homogeniser. 0.25 volume of buffer containing 10 mM Tris, pH 7.5, 0.5 mM MgCl₂ and 600 mM NaCl was added to the homogenate, after which the nuclei and unbroken cells were collected by centrifugation at 500 x g for 5 minutes. EDTA was added to the supernatant to a final concentration of 5 mM before centrifuging at 100,000 x g for 45 minutes. The resulting supernatant constituted the `cytosolic fraction´. The pellet was resuspended in lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 1.0% Triton X-100, and protease and phosphatase inhibitors as above) and centrifuged at 10,000 x g for 15 minutes. The supernatant from this step was termed the `membrane fraction´.

4.11. Immunofluorescence analysis (I, II)

Confluent cultures of RatER cells following treatments as indicated were washed twice with PBS, and then fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. The fixed cells were washed twice with PBS and extracted with acetone at -20 °C for 3 minutes. Cos-7 cells transfected with the indicated plasmids together with pEGFP-N1 as a marker for transfected cells were serum starved for 24 hours before fixation followed by washes with PBS and permeabilization with 0.25% Triton X-100. Immunofluorescent labelling of filamentous actin was carried out by incubation for 30 minutes at room temperature with 0.1 mg/ml of carboxytetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma). Following incubation with the fluorochrome-conjugated phalloidin, the cells were rinsed three times with PBS, and the samples were analysed with a Nikon Axiovert system at 100 x magnification. Cells expressing green fluorescent protein were studied under an excitation-emission filter for rhodamine to analyse actin fibre organisation. For immunostaining of vinculin, RatER cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, permeabilized in 0.25% Triton X-100 for 2 minutes and after blocking with 3% BSA the cells were incubated with anti-vinculin antibody for 1 hour. After washing with PBS, secondary TRITC-conjugated antibody was added to the cells. The cells were mounted on coverslips with fluoroguard and analysed under a microscope equipped for rhodamine fluorescence.
5. Results

5.1. Effects of EGF on Cas signalling (I)

5.1.1 Stimulation of the cells with EGF modulates the tyrosine phosphorylation status of Cas (I)

Previous studies have reported that the focal adhesion protein Cas provides an important integration site for various extra- and intracellular signals that appear to increase its tyrosine phosphorylation; transformation with Src and Crk oncogenes, ligand binding of integrins and stimulation of cells with growth factors NGF and PDGF are among these signals. By affecting the tyrosine phosphorylation status of Cas, these signals regulate the signalling events downstream. To investigate the putative role of EGF in the regulation of Cas-mediated signalling, we examined the effect of EGF on tyrosine phosphorylation of Cas in various EGF-responsive cell lines, including Rat-1 cells expressing the human EGF receptor (RatER), epidermoid carcinoma A431 cells and wild-type Rat-1 cells. The results described here are those obtained with RatER cells, but similar results were obtained with all cell lines tested. Cells were stimulated with different concentrations of EGF for 5 minutes, lysed and immunoprecipitated with an anti-Cas antibody that recognizes the extreme C-terminus of Cas, and does not cross-react with the Cas family members Efs/Sin or HEF-1/Cas-L. Compared to the unstimulated cells, there was a detectable increase in Cas tyrosine phosphorylation following treatment with low concentrations of EGF (0.5-5 ng/ml). In contrast, the phosphorylation content of Cas decreased sharply at high concentrations of EGF. Maximal reduction of tyrosine phosphorylation took place at 60 ng/ml of EGF, and was not further affected by treatment with higher concentrations. The tyrosine phosphorylation exhibited a bell-shaped dose-response curve. As a comparison, we studied tyrosine phosphorylation of p120 Ras-GAP, a previously identified substrate for the EGF receptor kinase (Margolis et al. 1989, Ellis et al. 1990). Tyrosine phosphorylation of p120 Ras-GAP increased in a dose-dependent manner in response to EGF stimulation, with no bell-shaped dose response curve.

Low concentrations of EGF stimulated a very rapid and transient tyrosine phosphorylation of Cas, as measured as a function of time. An increase in tyrosine
phosphorylation could be detected as early as 30-60 sec after the addition of 2 ng/ml EGF, reaching a maximum at 1-5 minutes. Thereafter, Cas tyrosine phosphorylation declined and remained above or at base-line levels for >4 hours. At high EGF concentrations (80 ng/ml), Cas tyrosine dephosphorylation was initially detected by 1 minute. There was a progressive decline in Cas phosphorylation that reached a maximum at 5-10 minutes of EGF treatment. The EGF-mediated decrease in Cas phosphorylation was transient and began to recover 30 minutes subsequent to the addition of 80 ng/ml of EGF. Following 60 minutes of EGF treatment, the tyrosine phosphorylation state of Cas was similar to that observed for the unstimulated control cells. During the periods of EGF treatment, there was no significant alteration in the amount of immunoprecipitated Cas.

5.1.2. EGF-induced tyrosine phosphorylation of Cas depends on the integrity of the actin cytoskeleton and the PI 3-kinase activity (I)

The formation of focal adhesions is accompanied by actin stress fibre formation, and actin cytoskeleton organisation occurs with the concomitant phosphorylation of focal adhesion proteins (Schwartz et al. 1995). To study if the changes in Cas tyrosine phosphorylation parallel changes in actin cytoskeleton organisation, quiescent cultures of RatER cells were treated with either 2 ng/ml or 80 ng/ml of EGF for various times and then fixed and stained with TRITC-conjugated phalloidin. Treatment of cells with a low concentration of EGF (2 ng/ml) resulted in a characteristic membrane ruffling and increased polymerisation of actin. In contrast, a high concentration of EGF (80 ng/ml) caused a dramatic disruption of actin stress fibres. The maximum effects of EGF on the actin cytoskeleton were observed after 5 minutes of EGF exposure, followed by a recovery to normal levels within 30-60 minutes. Immunostaining for vinculin indicated the presence of focal adhesion plaques in cells treated with a low concentration of EGF. In contrast, a high concentration of EGF stimulated the disruption of vinculin-containing focal adhesions.

To study whether disruption of the actin cytoskeleton could interfere with the increase in Cas tyrosine phosphorylation induced by low concentrations of EGF, the cells were pretreated with cytochalasin D for 2 hours. Cytochalasin D prevented Cas phosphorylation on tyrosine in response to 2 ng/ml of EGF in a dose-dependent manner. Tyrosine phosphorylation of Cas was completely blocked using a 2.0 μM concentration of cytochalasin D. This concentration was found to efficiently depolymerise the network of actin filaments in RatER-cells. Cytochalasin D treatment had no effect on the tyrosine phosphorylation of p120 Ras-GAP at any of the EGF concentrations that stimulated p120 Ras-GAP phosphorylation.

It has been shown that activation of PI 3-kinase is required for PDGF-stimulated membrane ruffling and tyrosine phosphorylation of focal adhesion proteins (Rankin & Rozengurt 1994, Wennstrom et al. 1994a, Wennstrom et al. 1994b, Wymann & Arcaro 1994, Abedi et al. 1995, Hawkins et al. 1995, Casamassima & Rozengurt 1997). PI 3-kinase is also known to become activated upon EGF treatment (Fantl et al. 1993). To examine the potential role of PI 3-kinase in EGF-stimulated membrane ruffling and tyrosine phosphorylation of Cas, quiescent RatER-cells were preincubated for 2 hours
with increasing concentrations of a PI 3-kinase inhibitor LY294002. Pretreatment with LY294002 inhibited Cas tyrosine phosphorylation induced by 2 ng/ml EGF for 5 minutes in a dose-dependent fashion. Maximum inhibition was achieved with 20 μM of LY294002; at the same concentration, LY294002 inhibited actin cytoskeleton reorganisation in EGF-treated RatER-cells. Pretreatment of cells with a structurally and functionally unrelated PI 3-kinase inhibitor wortmannin also induced a dose-dependent inhibition of the tyrosine phosphorylation of Cas in response to 2 ng/ml EGF. At 50 nM, wortmannin inhibited EGF-stimulated Cas phosphorylation by > 90%.

5.1.3. Treatment of the cells with EGF modulates the Cas-Crk complex formation (I)

To examine whether EGF-induced tyrosine phosphorylation of Cas could lead to the formation of a complex between Cas and Crk in intact RatER-cells, co-immunoprecipitation experiments with Cas and Crk were performed. Low concentrations of EGF (2 ng/ml) stimulated an association of Cas with Crk, whereas high concentrations of EGF (80 ng/ml), which stimulate dephosphorylation of Cas on tyrosine (see above), did not induce Cas-Crk interaction. To assess whether the complex formation between Cas and Crk depended on the integrity of the actin cytoskeleton, quiescent RatER-cells were pretreated for 2 hours with 2.0 μM cytochalasin D and then stimulated with 2 ng/ml of EGF for 5 minutes. Treatment of cells with cytochalasin D prevented the association of Cas with Crk induced by EGF. Therefore, Cas-Crk complex formation closely parallels the extent of the tyrosine phosphorylation of Cas in response to EGF stimulation.

5.2. Cas and Crk connect cellular stimuli to the Rac-JNK pathway (II)

EGF stimulation, integrin ligation and v-Src transformation are potent activators of c-Jun N-terminal kinases (JNKs) (Davis 1994, Miyamoto et al. 1995, Minden & Karin 1997). Small GTP-binding proteins Rac and Cdc42 are involved in initiating a cascade leading to their activation (Coso et al. 1995, Minden et al. 1995). Recently, Tanaka et al reported that JNK is activated in v-Crk transformed cells, and can be activated by transient overexpression of c-Crk and v-Crk (Tanaka et al. 1997), suggesting a possible role of Cas and Crk in the JNK pathway.

5.2.1. c-Crk activates the JNK pathway in an SH2- and SH3-dependent manner (II)
Although Crk has been suggested to be involved in integrin- and growth factor-mediated signalling pathways, the physiological role of Crk has remained largely unknown. To study the role of Crk in JNK activation, co-transfection experiments of COS-7 cells with the c-CrkII expression vector and a plasmid encoding a hemagglutinin (HA) epitope-tagged JNK1 were carried out. The kinase activity was measured using glutathione S-transferase (GST)-c-Jun (1-79) as the substrate. Co-expression of Crk resulted in efficient activation of JNK kinase activity which was measured in JNK immunoprecipitates. To study whether JNK activation results in c-Jun activation, HeLa cells were co-transfected with a GAL4-c-Jun (1-223) fusion protein containing the c-Jun activation domain, pFRLuc as the reporter gene, and with the c-CrkII expression vector. The activation of endogenous JNK by Crk resulted in stimulation of c-Jun transcriptional activity which was shown by induction of luciferase activity. As a control, overexpression of another adaptor protein, Grb2, failed to activate the JNK pathway.

To study if SH2 and SH3-domains of Crk are necessary in Crk-induced JNK activation, plasmids of mutant forms of Crk carrying either nonfunctional SH2 (c-CrkII-R38V), or nonfunctional SH3 (c-CrkII-W169L) domains were co-transfected with c-CrkII expression vector in COS-7 cells. Both mutated forms of Crk failed to induce JNK activation. Co-transfections of the same mutant forms of Crk with the GAL4-c-Jun (1-223) fusion protein and a pFRLuc reporter gene failed to stimulate the transcriptional activity of c-Jun.

5.2.2. Crk connects Cas to the JNK pathway (II)

To investigate the putative role of Cas in Crk-induced JNK activation, COS-7 cells were transiently transfected with an expression plasmid for Cas alone or together with a Crk expression plasmid. When Cas was expressed alone at a level that was 2-fold higher than the endogenous protein, corresponding increases in both the co-precipitation of Cas with Crk and in JNK kinase activation were measured. Co-transfection of Crk and Cas together resulted in higher JNK activation compared to expression of Cas or Crk alone. To investigate the significance of the Cas substrate domain in JNK activation, a mutant form of Cas with the substrate domain deleted (CasΔSD) which therefore is not capable of interacting with Crk, was expressed either alone or with Crk in COS-7 cells. CasΔSD expression did not induce JNK activation, and failed to potentiate the Crk-induced JNK activation. When the SH2-mutant form of Crk (c-CrkII-R38V), which is not capable of interacting with Cas, was expressed in COS-7 cells alone or together with wild-type Cas expression vector, no induction in JNK activation was measured.

Similar transfection experiments were carried out in HeLa cells with co-transfection of the GAL4-c-Jun (1-223) fusion protein and a pFRLuc reporter gene and luciferase activity was measured. c-Jun transcriptional activation was detected in the experiments where the induction in JNK activity was seen.

5.2.3. Rac is a downstream mediator for Cas- and Crk-induced JNK activation (II)
Cells that were transfected with an expression plasmid for Crk or Cas exhibited extensive membrane ruffling, as studied by staining the cells with rhodamine-conjugated phalloidin to visualise polymerised actin. The activated small GTP-binding protein Rac has been shown to induce membrane ruffle formation (Ridley et al. 1992), suggesting that Crk and Cas efficiently activate Rac. Expression of a dominant-negative form of Rac (Rac1N17) with Cas or Crk in COS-7 cells blocked membrane ruffling. Also, JNK activation was blocked when a dominant-negative form of Rac (Rac1N17) was co-transfected with expression vectors for Cas or Crk together with HA-JNK1 in COS-7 cells. As a control, co-transfection of cells with the dominant-negative Rap-1 (Rap1N17), another small GTP-binding protein, and with expression plasmids for Crk and HA-JNK1 had a negligible effect on JNK activation.

5.2.4. Cas links integrins to the JNK signalling pathway via c-Crk (II)

To investigate the putative role of Crk in mediating the cellular stimuli such as EGF stimulation, v-Src transformation and integrin ligation to JNK activation, Crk SH2- and SH3-mutants described above were used as specific dominant-negative inhibitors for Crk signalling (Matsuda et al. 1992, Tanaka et al. 1993, Matsuda et al. 1994, Tanaka et al. 1995). JNK activation was clearly detectable after transient expression of v-Src, stimulation of cells with EGF and attachment of cells to fibronectin. Expression of either the SH2- or the SH3-mutant of Crk in the cells blocked both the transcriptional activation of c-Jun and JNK catalytic activation in response to all these stimuli.

The role of Cas in connecting integrins, EGF receptor and v-Src via Crk to the JNK pathway was then studied. When the mutant form of Cas with the substrate domain deleted (CasΔSD) was expressed in COS-7 cells together with HA-JNK1, an integrin-mediated induction in JNK activation was blocked, whereas a negligible effect on v-Src or EGF-induced JNK activation was seen. Also, co-transfection of CasΔSD with the GAL4-c-Jun (1-223) fusion protein and a pFRLuc reporter gene in HeLa cells blocked integrin ligation induced c-Jun transcriptional activation, but had no effect in c-Jun transcriptional activation after transient transfection of cells with v-Src or after stimulation of the cells with EGF.

5.2.5. Role of Crk SH3-binding proteins in JNK activation (II)

To study the putative role of Crk SH3 domain-binding proteins Sos, C3G and DOCK180 in connecting the upstream stimuli to the Rac-JNK pathway, vectors coding for Sos, C3G and DOCK180 were expressed in COS-7 cells, and their ability to activate the JNK pathway was determined by performing a JNK kinase assay and by measuring c-Jun transcriptional activation. All three proteins were able to induce activation of the JNK pathway. Further, synergistic activation of JNK activity was detected by a JNK immunocomplex assay when Crk was co-expressed with DOCK180, but not with C3G or Sos. However, co-expression of Crk with C3G also demonstrated synergistic activation of
the JNK pathway when a c-Jun transcriptional activity assay was used to measure JNK activation. Co-expression of Sos with Crk did not have any effect on Crk-induced JNK pathway activation. When Sos or DOCK180 were co-expressed with dominant-negative Rac in COS-7 cells, inhibition of Sos- and DOCK180-induced JNK activation was observed. Co-expression of dominant-negative Rac with C3G did not have any effect on C3G-induced JNK activation.

5.3. Role of Cbl in integrin signalling (III)

5.3.1. Cell adhesion induces tyrosine phosphorylation of Cbl (III)

In order to study adhesion-dependent signalling events in a myeloid cell system, macrophage cell lines IC-21, P388D1 and RAW 264.7 were used and similar results were obtained with all three lines. The results indicated here are from experiments with the IC-21 cell line. IC-21 cells were either kept in suspension or plated on dishes coated with fibronectin. Cell adhesion to fibronectin resulted in elevated tyrosine phosphorylation of proteins of 150-170 kDa, 115-135 kDa and 70-85 kDa in molecular weight. Immunoprecipitation of samples from suspended and adherent IC-21 cells with anti-Cbl antibodies followed by immunoblotting analysis with anti-phosphotyrosine antibodies, revealed that the proto-oncogene product Cbl was one of the phosphoproteins in the 115-135 kDa range. When immunoprecipitated from suspended cells, Cbl exhibited a very low level of tyrosine phosphorylation. An increase in tyrosine phosphorylation of Cbl was seen in cells plated on fibronectin, vitronectin, anti-α5β1 or anti-αvβ3 antibodies. Cell adhesion to polylysine, to which cells can adhere in a nonspecific fashion, or on dishes coated with anti-MHC antibodies did not effect the tyrosine phosphorylation of Cbl.

Cbl tyrosine phosphorylation during cell adhesion was gradual and persistent as measured as a function of time. Cbl contained little phosphotyrosine at time zero, but tyrosine phosphorylation was detectable after 10 minutes of adhesion. After 20 minutes, a clear increase in Cbl phosphorylation was observed in cells plated on fibronectin; at this time point, most of the cells had adhered to fibronectin, but had not yet fully assumed a flat, spread morphology. Maximal tyrosine phosphorylation of Cbl occurred at 40 minutes after plating when macrophages appeared maximally spread on fibronectin. After 90 minutes, the phosphorylation slowly declined and remained unchanged after 5 hours of plating under serum-free conditions. CSF-1 stimulation, which was earlier shown to induce rapid and transient tyrosine phosphorylation of Cbl in macrophages, was used as a comparison (Wang et al. 1996). Anti-phosphotyrosine immunoblotting of anti-Cbl immunoprecipitates from cell lysates prepared at various times after 25 nM CSF-1 stimulation of IC-21 cells showed that Cbl is rapidly and transiently (peak at 1-3 minutes) tyrosine-phosphorylated.

5.3.2. Intact cytoskeleton is required for the integrin-induced tyrosine phosphorylation of Cbl (III)
To determine whether the integrity of the cytoskeleton is necessary for cell adherence to stimulate tyrosine phosphorylation of Cbl, the spreading of cells plated onto a fibronectin substratum was inhibited with cytochalasin D; cytochalasin D treatment has been shown to prevent integrin-mediated tyrosine phosphorylation of Cas, FAK and tensin (Bockholt & Burridge 1993, Nojima et al. 1995, Vuori & Ruoslahti 1995). Cytochalasin D prevented cell spreading and tyrosine phosphorylation of Cbl in a dose-dependent manner. At a cytochalasin D concentration of 0.05 μM, no effect was observed on cell spreading or on tyrosine phosphorylation of Cbl. Cytochalasin D at a concentration of 0.25 μM clearly decreased the integrin-induced tyrosine phosphorylation of Cbl; this decrease correlated with the inhibition of cell spreading. A complete inhibition of both cell spreading and adhesion-induced Cbl phosphorylation was observed when cells were treated with 1.0 μM of cytochalasin D. Cytochalasin D treatment at a concentration of 1.0 μM did not affect the tyrosine phosphorylation of Cbl following CSF-1 stimulation.

5.3.3. Interactions of Cbl with SH2 and SH3 domain-containing signalling proteins (III)

To test whether SH2 domain-containing proteins would bind to Cbl in an adhesion-dependent manner, binding experiments with various GST-SH2 fusion proteins were conducted. The SH2 domains of Crk, PI 3-kinase and Src bound to Cbl in lysates of IC-21 cells that had been plated on fibronectin. No SH2 domain binding to Cbl was seen in cell lysates prepared from suspended cells or from cells plated on polylysine. No interaction was detected between Cbl and the SH2 domain of Shc. Consistent with earlier observations, The GST-SH3 domains of Src and PI 3-kinase, but not of Crk, bound to Cbl in IC-21 cell lysates. Unlike the SH2 domain interactions described above, the SH3 domain interactions were adhesion-independent, since the GST-SH3 domains precipitated Cbl equally well from suspended and adherent cells.

Co-immunoprecipitation experiments in suspended and adherent cells were performed with antibodies against Crk, Src and PI 3-kinase followed by immunoblotting with anti-Cbl antibodies. Cbl co-precipitated with Src and PI 3-kinase from fibronectin-adherent IC-21 cells. Little co-precipitation was detected in samples prepared from suspended IC-21 cells. Likewise, Src and PI 3-kinase, but not Crk, were detected in anti-Cbl antibody immunoprecipitates from adherent, but not from suspended cells (see later). No co-precipitation between Crk and Cbl was detected under these experimental conditions.

To determine whether the association between Cbl and Src and Cbl and PI 3-kinase was direct, Cbl was immunoprecipitated from suspended and fibronectin-adherent IC-21 cells, and subjected to an overlay assay using GST-SrcSH2, GST-SrcSH3, GST-PI 3-kinase-SH2 and GST-PI 3-kinase-SH3 fusion proteins as probes. A 120 kDa protein was detected by all the fusion proteins on the lanes of anti-Cbl precipitates from adherent cells; SH3 domain fusion proteins recognized the 120 kDa protein also from suspended cells. The 120 kDa protein had the same mobility as Cbl detected by re-probing of the membranes with anti-Cbl antibody. No protein was detected when control precipitates
with irrelevant antibodies were probed with the fusion proteins, or when anti-Cbl immunoprecipitates were probed with GST-ShcSH2.

5.3.4. Enzymatic activity of Src and the PI 3-kinase associate with Cbl in response to cell adhesion (III)

To examine if Src and PI 3-kinase are enzymatically active in Cbl signalling complexes, the \textit{in vitro} kinase activity and PI 3-kinase activity were measured. Anti-Cbl immunoprecipitates from suspended cells associated with low tyrosine kinase activity. Tyrosine kinase activity in Cbl immunoprecipitates was markedly increased upon integrin-mediated cell adhesion on fibronectin whereas no increase in tyrosine kinase activity was observed in samples prepared from cells plated on polylysine. Re-precipitation experiments identified Cbl as one of the phosphorylated 120 kDa proteins and Src as one of the 60 kDa proteins present in the \textit{in vitro} kinase assay complex from fibronectin-adherent cells. To determine whether Src exhibits kinase activity in the Cbl complex, Cbl immunoprecipitates were subjected to \textit{in vitro} kinase assay in the presence of enolase, a classic Src family kinase substrate. These experiments demonstrated 5-fold increased kinase activity towards enolase in Cbl immunoprecipitates from adherent cells compared to immunoprecipitates from suspended cells.

An \textit{in vitro} lipid kinase assay showed that PI 3-kinase activity was negligible in anti-phosphotyrosine and anti-Cbl immunoprecipitates in IC-21 cells kept in suspension in the absence of any growth factor stimulation; neither did replating of cells onto polylysine stimulate the PI 3-kinase activation. Plating of cells on fibronectin or on anti-integrin antibodies rapidly stimulated the PI 3-kinase activity in IC-21 cells; anti-phosphotyrosine and anti-Cbl immunoprecipitates from cells plated on fibronectin and on anti-α5β1 antibodies demonstrated a substantial level of PI 3-kinase activity. Unrelated control antibody did not immunoprecipitate any PI 3-kinase activity from suspended or adherent IC-21 cells. As a positive control, CSF-1 stimulation resulted in an association of PI 3-kinase activity with the Cbl immunoprecipitates. Maximal PI 3-kinase activity association with Cbl was detected after stimulation of the cells for one minute with CSF-1. The time course correlated with the time course of tyrosine phosphorylation of Cbl observed in response to CSF-1 stimulation (see earlier).

5.3.5. Cbl relocates to the plasma membrane during cell attachment (III)

To further evaluate the biological responses of Cbl to integrin stimulation, the effect of cell adhesion on Cbl subcellular localisation was studied. Cells adherent on polylysine or on fibronectin were fractionated as described under Materials and methods. Antiserum against Cbl was used to immunoadsorb Cbl from both cytosol and membrane fractions, and immunoprecipitates were resolved by SDS-PAGE and probed with antibodies against Cbl and against phosphotyrosine. In both polylysine- and fibronectin-adherent cells, the majority of the cellular pool of Cbl was found in the cytoplasmic fraction. However, a 5-
fold increase in the concentration of Cbl in the membrane fraction was observed in cells plated on fibronectin compared to cells plated on polylysine. Little tyrosine phosphorylation on Cbl was detected in both cytosol and membrane fractions prepared from cells plated on polylysine, whereas plating of cells on fibronectin stimulated an increase in tyrosine phosphorylation of Cbl in both the cytosolic and membrane fractions.

5.4. Oncogenic forms of Cbl induce anchorage independent cell growth (IV)

5.4.1. Protein tyrosine phosphorylation in suspended and adherent Cbl-transfected cells (IV)

To investigate whether tyrosine phosphorylation of the oncogenic forms of Cbl is regulated in an integrin ligand binding-dependent manner, previously established NIH 3T3 fibroblast transfectants expressing either wild-type human Cbl (c-Cbl) or three mutant forms of Cbl: Y368del, Y371del and 70Z/Cbl (Andoniou et al. 1994) were used. NIH 3T3 transfectants expressing mutant Cbl proteins exhibited a transformed phenotype in culture whereas the wild-type Cbl-expressing cells remained untransformed (Andoniou et al. 1994). Overall, tyrosine phosphorylation of proteins in suspended c-Cbl cells was negligible. Similar to the parental NIH 3T3 cells (Guan et al. 1991, Burridge et al. 1992), integrin-mediated cell attachment to fibronectin resulted in elevated tyrosine phosphorylation of proteins of 115-135 kDa, 70-80 kDa and 55-60 kDa in molecular weight. By contrast, cells expressing mutant forms of Cbl displayed increased tyrosine phosphorylation of intracellular proteins when kept in suspension compared to cells expressing wild-type Cbl; this was most notable with protein(s) of 115-135 kDa in molecular weight. Integrin-mediated cell attachment resulted in a further increase in tyrosine phosphorylation of cellular proteins and, when compared to cells expressing wild-type Cbl, proteins of 115-135 kDa, 70-80 kDa and 55-60 kDa in molecular weight were hyperphosphorylated in cells expressing mutant forms of Cbl.

When Cbl was immunoprecipitated from suspended c-Cbl-expressing cells, Cbl exhibited a very low level of tyrosine phosphorylation. An increase in tyrosine phosphorylation of c-Cbl was seen in cells plated on fibronectin. These results are indistinguishable from those reported for the endogenous Cbl in myeloid cells (III). In contrast, tyrosine phosphorylation of mutant forms of Cbl was readily detectable in cells kept in suspension; cell attachment to fibronectin did not result in a further increase in the tyrosine phosphorylation of the oncogenic Cbl-proteins. Compared to the tyrosine phosphorylation observed for wild-type Cbl in adherent cells, mutant forms of Cbl were hyperphosphorylated in suspended and attached cells. The experiments were carried out with and without prior serum starvation of the cells, and no differences were found in the results between these two conditions.

By using specific antibodies against focal adhesion proteins Cas and focal adhesion kinase FAK, these proteins were found to be among the 115-135 kDa proteins that become tyrosine-phosphorylated in an adhesion-dependent manner in c-Cbl-expressing
cells and are hyperphosphorylated in adherent, mutant Cbl-expressing cells. Neither one of these proteins, however, demonstrated significant adhesion-independent tyrosine phosphorylation in cells expressing oncogenic forms of Cbl. The identity of the protein(s) which, in addition to the oncogenic forms of Cbl, exhibit adhesion-independent tyrosine phosphorylation in these cells remains to be determined.

5.4.2. Interactions of different forms of Cbl with intracellular signalling proteins in suspended and adherent cells (IV)

Binding experiments with various GST-SH2 fusion proteins demonstrated that wild-type Cbl expressed in 3T3 cells bound to SH2 domains in an adhesion-dependent manner. The SH2-domain of Src, the p85 subunit of PI 3-kinase and the adapter protein Crk bound to wild-type Cbl in lysates of cells that had been plated on fibronectin but not of cells that had been kept in suspension. In contrast, the mutant forms of Cbl readily interacted with the SH2 domains in both suspended and adherent cells. Further, we found that PI 3-kinase activity is associated with oncogenic forms of Cbl in both suspended and attached cells. In contrast, PI 3-kinase activity was found to be associated with wild-type c-Cbl complexes in an adhesion-dependent manner. Total PI 3-kinase activity paralleled the Cbl-associated PI 3-kinase activity; PI 3-kinase activity was negligible in anti-phosphotyrosine immunoprecipitates in suspended cells expressing wild-type Cbl, whereas the activity was readily detectable in the immunoprecipitates prepared from attached wild-type Cbl-expressing cells and from suspended and attached mutant Cbl-expressing cells. Similarly, tyrosine kinase activity was detected in Cbl immunoprecipitates from both suspended and adherent cells expressing oncogenic forms of Cbl. By contrast, tyrosine kinase activity associated with wild-type c-Cbl was markedly increased upon integrin-mediated cell adhesion to fibronectin.

5.4.3. Growth characteristics of the Cbl-transfected cells (IV)

To further analyse how the oncogenic forms of Cbl alter signalling pathways, proliferation of the various Cbl-transfectants was studied. First, the ability of the various transfectants to grow in soft agar was tested. When put in semi-solid medium containing 10% serum, 3T3 cells transfected with oncogenic forms of Cbl rapidly formed large colonies. After 14 days, 25-30% of the cells harbouring oncogenic forms of Cbl formed colonies, as compared with <0.5% for control and wild-type Cbl-transfected 3T3 cells. To determine if the transformed cells showed a reduced requirement for growth factors, the cells were tested for growth in soft agar containing 0.5% serum. Interestingly, transfectants expressing oncogenic forms of Cbl showed no detectable colony formation (<0.5%) in semi-solid medium containing 0.5% serum.

The parental 3T3 cells and transfecant cell lines were plated on tissue culture dishes in medium with either 0.5% or 10% of serum to study the growth factor independency for proliferation. No indication of serum-independent growth was detected, as all cell lines
failed to proliferate in medium containing 0.5 % serum. Both control and transfected cells
died in serum-free medium. Parental 3T3 cells and transfected cell lines proliferated at a
similar rate in the presence of 10% serum, with a doubling-time for proliferation being 22
hours. Interestingly, parental NIH 3T3 cells and c-Cbl-expressing cells appeared to reach
saturation in their growth by day 8 under our assay conditions. In contrast, cells
expressing oncogenic forms of Cbl continued to proliferate beyond this point. Parental
3T3 cells, c-Cbl cells and transfectants expressing oncogenic forms of Cbl had similar
proliferation rates at all serum concentrations tested (2-10%). Similar results were
obtained when the DNA synthesis rate of various transfectants was studied by measuring
[^3H]thymidine incorporation.
6. Discussion

Cell adhesion is of fundamental importance for both developing and adult tissues; it guides several important cellular functions. Aberrations in the adhesion-dependent signalling pathways may lead to severe pathological disorders. In order to understand and treat these clinical manifestations, we need to understand the intracellular events underlying these disorders. The main goal of the present research was to study the adhesion-dependent signalling pathways in fibroblasts and in hematopoietic cells, concentrating on tyrosine phosphorylation events. Two important signalling proteins were studied; a known integrin signalling protein, p130\textsuperscript{Cas}, in fibroblasts and a signalling protein, p120\textsuperscript{Cbl}, which was identified in these studies as the main tyrosine-phosphorylated protein following integrin stimulation in cells of myeloid lineage. Interestingly, these both proteins share several characteristics; they are both docking proteins, they become tyrosine-phosphorylated following integrin stimulation in cells of myeloid lineage. Interestingly, these both proteins share several characteristics; they are both docking proteins, they become tyrosine-phosphorylated following integrin- and growth factor receptor stimulation and are well suited for signal transduction judged by their structure. In the first part of the discussion I will concentrate on signalling pathways mediated by Cas, and in the latter part on the signalling events involving Cbl.

6.1. Cas mediates signals from different cellular stimuli

Cas is tyrosine-phosphorylated upon stimulation of cells with several growth factors, but its exact role in growth factor signalling remains unclear. The present work demonstrates that EGF stimulation of cells results in an unusual bell-shaped dose response curve of tyrosine phosphorylation of Cas (I). Previously, EGF stimulation of cells has been shown to induce a bell-shaped dose response curve of tyrosine phosphorylation of FAK, which in many cases closely parallels that of Cas phosphorylation (Rankin \textit{et al.} 1996). However, no other substrates have been identified that would exhibit bell-shaped dose response curves in EGF-induced tyrosine phosphorylation. Recently, PDGF stimulation (Casamassima & Rozengurt 1997) has been shown to induce a bell-shaped dose-response curve of tyrosine phosphorylation of Cas. These findings suggest that PDGF and EGF receptor stimulation may connect to similar intracellular signalling pathways that induce
tyrosine phosphorylation of the focal adhesion proteins Cas and FAK, the pathways to other substrates being distinct from these.

Several stimuli that induce tyrosine phosphorylation of Cas also induce a rapid increase in stress fibres in the focal contacts; these stimuli include integrin-mediated cell adhesion, neuropeptides, lysophosphatidic acid and sphingosylphosphorylcholine (Burridge et al. 1988, Rankin et al. 1994, Seufferlein & Rozenburg 1995). In the present work, an increase in actin stress fibre polymerisation paralleled the EGF-induced tyrosine phosphorylation of Cas. An intact actin cytoskeleton was required for Cas phosphorylation, since tyrosine phosphorylation was blocked by cytochalasin D that disrupts the cytoskeletal organisation (I). A similar situation has been observed when Cas phosphorylation was induced by bombesin, low concentrations of PDGF or integrin-mediated cell adhesion (Nojima et al. 1995, Vuori & Ruoslahti 1995, Casamassima & Rozenburg 1997). These findings suggest that proper actin cytoskeleton organisation is required to bring together the kinases and other possible signalling components involved in Cas tyrosine phosphorylation upon EGF stimulation and other stimuli.

The effects on stress fibres and focal adhesions by extracellular stimuli are likely to be mediated by the small G-proteins Rac and Rho (see Hall 1994). A recent work (Casamassima & Rozenburg 1997) demonstrated PDGF-induced Cas tyrosine phosphorylation to be mediated through PI-3K and Rac. In the present work the signalling cascade may be similar, since the EGF-induced Cas tyrosine phosphorylation was blocked by PI-3K inhibitors (I).

When cells are stimulated with insulin (Knight et al. 1995) or with high concentrations (10ng/ml or more) of EGF (I), the dephosphorylation seen in focal adhesion proteins correlates with the focal adhesion disassembly and actin stress fibre breakdown. The mechanism for Cas tyrosine dephosphorylation may involve activation of tyrosine phosphatases upon cellular stimuli. PTP1B and PTP-PEST have been shown to interact and dephosphorylate Cas (Garton et al. 1996, Liu et al. 1996). Dephosphorylation may also take place by inactivation of tyrosine kinases; C-terminal Src kinase Csk is involved in insulin's regulation of the phosphorylation of focal adhesion proteins possibly through inactivation of the kinase activity of the Src family kinases (Tobe et al. 1996).

Previously, it has been shown that several factors that contribute to tyrosine phosphorylation of Cas can induce an SH2 domain-mediated association of Crk with Cas (Casamassima & Rozenburg 1997, Vuori et al. 1996). Here it was demonstrated that low concentrations of EGF induce Cas-Crk complex formation and that this interaction is dependent on the intact actin cytoskeleton (I). The interaction between Cas and Crk may be important in regulating the subcellular localization of Crk or the downstream effectors in EGF signal transduction.

The present findings that expression of c-Crk results in JNK activation confirm earlier observations (Tanaka et al. 1997). Results reported here further indicate that endogenous c-Crk has a physiological role in connecting a number of cellular stimuli to the JNK pathway via the small guanine nucleotide binding protein Rac (II). These stimuli include EGF receptor activation, integrin-mediated cell adhesion and transformation by v-Src. It was also demonstrated that tyrosine-phosphorylated Cas is a necessary intermediate in the pathway leading from integrins to the JNK activation via Crk, whereas a different mechanism may be connecting EGF receptor activation and v-Src expression.
to JNK activation (II). Rac activation downstream of Cas-Crk complex is likely to be biologically important since it has also been shown to mediate cell migration. Klemke et al. demonstrated that overexpression of Cas and Crk results in enhanced cell migration that can be blocked by a dominant-negative form of Rac (Klemke et al. 1998). Expression of dominant-negative forms of Cas and Crk were able to block both integrin-mediated haptotactic migration and cytokine induced migration. These findings together suggest that Cas-Crk interaction may be important in mediating signalling pathways from different stimuli that affect a variety of cell functions.

The role of Cas or another Crk SH2 domain-binding protein may be to localise Crk to the plasma membrane, recruiting Crk SH3 domain-bound guanine exchange factors C3G (Tanaka et al. 1994), Sos (Matsuda et al. 1994), or perhaps DOCK180 (Hasegawa et al. 1996) to activate Rac. Sos and DOCK180 have been shown to associate directly with and activate Rac (Kiyokawa et al. 1998a, Nimnual et al. 1998). Other reports have highlighted the role of C3G in the activation of JNK, however, Rac seems not to have any role in this pathway, since dominant-negative Rac did not block C3G induced JNK activation (Tanaka et al. 1997, Tanaka & Hanafusa 1998). Increasing evidence points to the role of DOCK180 as the downstream effector of Cas-Crk signalling. Integrin ligation results in association of DOCK180 with Cas-Crk complex and its co-expression with Cas and Crk induces cell spreading and accumulation of DOCK180 in focal adhesions (Kiyokawa et al. 1998b). Association and activation of Rac by DOCK180 was shown to be necessary for the integrin induced cell spreading in fibroblasts (Kiyokawa et al. 1998b). Our results show that Rac is a necessary intermediate in Crk-induced JNK activation and that dominant-negative Rac is an efficient inhibitor of DOCK180 and Sos-induced JNK activation (II). This suggests that DOCK 180 or Sos (rather than C3G) may connect Cas and Crk to the Rac-JNK pathway.

The recent Cas knock-out work by Hirai and coworkers gives insight into what the biological role of Cas might be (Honda et al. 1998). Cas-deficient mice died in utero and showed growth retardation and anomalies in the cardiovascular system, indicating that Cas is essential for embryogenesis. Cas-deficient fibroblasts isolated from the embryos in turn demonstrated thin, short and disorganized actin filaments, changes that could be restored by re-expression of Cas in these cells. Moreover, the Cas-deficient fibroblasts did not exhibit a transformed phenotype when transfected with constitutively active Src, and neither did these cells develop colonies when grown on soft agar. In Cas-deficient cells, Src transfection resulted in incomplete accumulation of actin filaments to podosomes, which are focal contact-like structures in transformed cells. This suggests that Cas is required for Src-induced transformation possibly through regulating actin cytoskeleton organisation. In the present study, no evidence was found that v-Src transformation induced Rac-JNK activation would be mediated through the Cas-Crk complex (II), suggesting that another pathway through Cas is necessary for v-Src induced transformation.
Fig. 5. A model for the integrin signalling pathways mediated by p130Cas in fibroblasts. It should be noted, that growth factors also modulate this pathway, the response being dependent on the concentration and duration of the growth factor stimulus used.
6.2. Cbl as a mediator of adhesion-dependent signals

Previous studies have reported the tyrosine phosphorylation of Cbl to occur in response to a number of stimuli, many of which influence cell morphology, locomotion, growth and differentiation. In the present study, Cbl was demonstrated to be a predominant phosphorylated component in macrophages upon integrin-mediated cell adhesion (III). In contrast to the gradual and persistent tyrosine phosphorylation of Cbl observed during cell adhesion (III), CSF-1 stimulation resulted in a rapid and transient tyrosine phosphorylation as also reported previously (Wang et al. 1996). These results suggest that integrin- and growth factor mediated signalling pathways resulting in Cbl phosphorylation are mechanistically different. Similar to tyrosine phosphorylation of FAK, tensin and Cas in other cells (Bockholt & Burridge 1993, Nojima et al. 1995, Vuori & Ruoslahti 1995), tyrosine phosphorylation of Cbl requires the presence of an intact cytoskeleton, since the adhesion-induced tyrosine phosphorylation of Cbl was prevented by cytochalasin D treatment (III).

The structure of Cbl suggests that it is a signal assembly protein; Cbl has been shown to interact with several SH2 and SH3 domain-containing proteins, including Src family kinases, PI 3-kinase and Crk. In the studies reported here SH3 domains of Src and PI 3-kinase bound Cbl in an adhesion-independent manner, whereas the interactions between Cbl and the SH2 domains of Crk, Src and PI 3-kinase required integrin-mediated cell adhesion (III). Co-precipitation of Cbl with Src and PI 3-kinase was observed only in adherent cells, suggesting that a stable complex formation between these proteins may require the SH2 domain binding to phosphorylated Cbl. No co-precipitation of Cbl and Crk was detected probably due to the experimental conditions.

An activated Src co-precipitated with Cbl, which suggests that Src might be responsible for the tyrosine phosphorylation of Cbl during integrin-mediated ligand binding. Enolase was used as a substrate in the in vitro tyrosine kinase assays; since enolase can be phosphorylated not only by Src, but also by other members of the Src family, it is possible that some of the other family members may contribute to the tyrosine kinase activity observed in Cbl complexes following integrin ligand binding. Src family members Hck, Fgr and Lyn are known to be expressed in myeloid cells (Lowell & Soriano 1996). Fgr and Lyn are activated upon integrin binding in human neutrophiles and associate with Cbl in vivo in macrophages (Berton et al. 1994, Yan et al. 1995, Meng & Lowell 1998). In Hck, Fgr and Lyn triple-mutant macrophages, Cbl phosphorylation is blocked and its membrane translocation upon cell adhesion is impaired (Meng & Lowell 1998). The interaction of Src family members with Cbl may lead to Cbl phosphorylation by enzymatic activation of the kinase through a mechanism similar to that proposed to occur during FAK-Src interaction upon integrin ligand binding (Schaller et al. 1994, Calalb et al. 1995). However, the molecular events leading to the initial step of the tyrosine phosphorylation of Cbl are currently unknown.

Activation of PI 3-kinase has been connected to a number of biological effects, such as the mitogenic effects of certain growth factors, changes in actin rearrangement, antiapoptotic events and growth factor-mediated membrane ruffling and chemotactic migration (for reviews, see Kapeller & Cantley 1994, Toker & Cantley 1997). In the present study, an in vivo association of Cbl with PI 3-kinase upon integrin ligand binding was demonstrated, and further, PI 3-kinase activity associated with Cbl in an adhesion-
dependent manner (III). Meng et al showed that Hck, Lyn and Fgr triple-mutant macrophages have reduced Cbl- and cytoskeletal-associated PI 3-kinase activity suggesting that Src family kinases are necessary in Cbl phosphorylation leading to Cbl-PI 3-kinase complex formation, membrane localisation and kinase activation (Meng & Lowell 1998). PI 3-kinase is known to be activated when one or both of the SH2 domains in the p85 subunit of this enzyme bind to tyrosine-phosphorylated proteins that contain Y-X-X-M motifs (Songyang et al. 1993). In the present study the N-terminal SH2 domain of p85 PI 3-kinase bound to Cbl in an adhesion-dependent manner; Cbl has two Y-X-X-M motifs (Langdon 1995), that can serve as binding sites for the SH2 domains of PI 3-kinase. These results suggest that one functional consequence of Cbl tyrosine phosphorylation upon integrin ligand binding is the catalytic activation of PI 3-kinase. As Cbl associates with cell surface receptors, it may also serve as an adaptor to recruit PI 3-kinase to activated receptors, in particular to those that lack PI 3-kinase binding sites such as EGF receptor and the lymphocyte antigen receptors (Fukazawa et al. 1995, Meisner & Czech 1995, Soltsoff & Cantley 1996). Recently, Cbl was shown to be required for PI 3-kinase-dependent activation of integrins by CD28 receptor in T-cells (Zell et al. 1998), suggesting that Cbl might also regulate integrin activation by recruiting PI 3-kinase to the integrin receptor. Inhibition of PI 3-kinase activity or treatment of cells with antisense Cbl oligonucleotides inhibited macrophage spreading on fibronectin (Meng & Lowell 1998) also suggesting a potential role for Cbl in regulating integrin function. Thus, the Cbl-PI 3-kinase interaction may be involved in mediating several integrin-dependent cell functions.

The ability of the naturally occurring and genetically engineered mutants of Cbl to transform NIH 3T3 fibroblasts was used to study the possible role of adhesion and growth factors in Cbl-induced tumourigenesis. Results of this work indicate that oncogenic forms of Cbl abrogate the adhesion requirement, but not the growth factor requirement for proliferation (IV). This suggests a novel mechanism of oncogenesis by mutant forms of Cbl to induce constitutive activation of an integrin-dependent signalling pathway. By implication, these studies identify a potential physiological role for the cellular form of Cbl in intracellular signalling of integrins.

Previous studies have shown that oncogenic mutants of Cbl are hyperphosphorylated in cells growing as a monolayer (Andoniou et al. 1994, Bonita et al. 1997). The results of the present study demonstrate that this tyrosine phosphorylation is adhesion-independent and confirm the finding that oncogenic forms of Cbl in attached cells are hyperphosphorylated compared to the cellular form of Cbl (IV). A clear correlation was demonstrated between the constitutive tyrosine phosphorylation of Cbl and anchorage-independent growth (IV) suggesting that tyrosine phosphorylation provides a biochemical mechanism for Cbl's transforming activity. The molecular mechanism of how cells become anchorage-independent is unknown, but one explanation may be that oncogenic proteins promote constitutive complex formation between Cbl and SH2 domain-containing signalling proteins, such as Src and PI 3-kinase, leading to the constitutive activation of these pathways. Indeed, the results here demonstrated an anchorage-independent association of the oncogenic mutants of Cbl with SH2 domains of Src, PI 3-kinase and Crk and an increase in tyrosine kinase activity and PI 3-kinase activation in cells expressing oncogenic forms of Cbl, whereas the activation of these kinases in non-transformed cells was anchorage-dependent (III, IV).
Increasing evidence suggests a physiological role for c-Cbl as a regulator of tyrosine kinases. Our finding that oncogenic forms of Cbl are constitutively associated with tyrosine kinase activity and that they induce hyperphosphorylation of several intracellular proteins further supports this view (IV). Oncogenic forms of Cbl associate with EGF and PDGF receptors (Bonita et al. 1997, Thien & Langdon 1997) and the 70Z/Cbl induce the transcriptional activation of the nuclear factor of activated T-cells (NFAT) (Liu et al. 1997). These results suggest that oncogenic mutants have lost their ability to function as negative regulators, but still provide binding sites for complexes that mediate growth-stimulating signals. Recent Cbl knock-out work suggests a role for c-Cbl as a negative regulator of growth-stimulating signals. Cbl knock-out mice displayed marked lymphoid hyperplasia, altered T-cell receptor expression, primary splenic extramedullary hematopoiesis and increased ductal density and branching in mammary fat pads (Murphy et al. 1998). In the present study the cells expressing oncogenic forms of Cbl proliferated beyond the point where c-Cbl expressing cells ceased to grow (IV). This finding is consistent with the hyperplastic changes seen in Cbl knock-out mice, again suggesting that one biological function of c-Cbl may be the negative regulation of growth stimulating signals.

This study provides evidence that cellular transformation by oncogenic forms of Cbl occurs mainly by activation of an integrin-dependent, rather than growth factor-dependent pathway, since oncogenic forms of Cbl abrogated the adhesion-, but not the growth factor-requirement for proliferation (IV). This is in contrast to a behavior displayed by many other oncogenes, including v-Ras, which render cells both anchorage- and serum-independent. Previous results in experimental cell systems have indicated that the constitutively activated form of FAK (Frisch et al. 1996) and oncogenic forms of Rho exchange factors (Schwartz et al. 1996) also induce anchorage-independent but serum-dependent growth, and make cells tumourigenic. Thus, these results suggest that anchorage-independence and serum-independence can be separated, which may be an important concept for understanding tumourigenesis.

Recent findings indicate that inadvertent activation of the integrin signalling pathway, without concomitant activation of the growth factor signalling, may truly contribute to human malignancies; Renshaw et al. found that the human leukemia oncogene bcr-abl abrogates the anchorage-requirement but not the growth factor-requirement for proliferation (Renshaw et al. 1995). Cbl is the major tyrosine-phosphorylated protein in in bcr-abl-transformed cells (Andoniou et al. 1994), suggesting a possible role for Cbl in mediating bcr-abl induced tumourigenesis. It should be noted, however, that present studies, as well as those by Renshaw et al., utilised NIH 3T3 cells as the experimental model system; fibroblasts are not the targets of bcr-abl in vivo and neither do 3T3 cells express detectable levels of endogenous Cbl protein. Findings that both bcr-abl and oncogenic forms of Cbl abrogate anchorage-requirement for growth are consistent with many of the clinical manifestations of human chronic myelogenous leukemia (Renshaw et al. 1995; for a review, see Verfaillie et al. 1997).

Results of the present study suggest that a physiological role of cellular Cbl is to be a component of an integrin-dependent growth regulatory pathway. Assuming that the oncogenic forms of Cbl act in a dominant-positive manner, these findings would suggest that the role of endogenous Cbl is to support integrin-dependent cell proliferation. It is possible that the mutant forms of Cbl integrate into and activate the integrin pathway by
assuming a new function as a result of the mutations. However, oncogenic mutants of Cbl might function by a dominant-negative mechanism, inhibiting the function of the endogenous Cbl or, in 3T3 cells, a Cbl-like molecule. This explanation would lead to the conclusion that the function of cellular Cbl in the integrin pathway is to mediate growth-inhibitory signals. Cbl may be a component of multiple signalling pathways; in addition to integrin-mediated cell attachment, tyrosine phosphorylation of cellular Cbl and binding to SH2 domain-containing molecules has been reported to occur in response to a number of other stimuli. Although no evidence was found that constitutively tyrosine-phosphorylated forms of Cbl would significantly affect growth factor signalling, it is obvious that Cbl is also an important component in these pathways. Further genetic and biochemical studies are required to fully discern the multiple important roles Cbl may have in intracellular signalling pathways.

Fig.6. A model for the integrin signalling pathway involving p120<sup>CM</sup> in hematopoietic cells.
7. References


Blake, T.J., Shapiro, M., Morse, H.C.d. & Langdon, W.Y. (1991) The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene 6: 653-657.


