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CONFORMATIONAL REGULATION OF α2β1 INTEGRIN IN LIGAND BINDING

by

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To my family

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Conformational Regulation of α2β1 Integrin in Ligand Binding
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ABSTRACT

Integrins are heterodimeric cell adhesion receptors involved in cell-cell and cell-extracellular matrix (ECM) interactions. They transmit bidirectional signals across the cell membrane. This results in a wide range of biological events from cell differentiation to apoptosis. α2β1 integrin is an abundant collagen receptor expressed on the surface of several cell types. In addition to ECM ligands, α2β1 integrins are bound by echovirus 1 (EV1) which uses α2β1 as a receptor to initiate its life cycle in the infected cell. The aim of this thesis project was to provide further insight into the mechanisms of α2β1 integrin ligand recognition and receptor activation.

Collagen fibrils are the principal tensile elements of the ECM. Yet, the interaction of α2β1 integrin with the fibrillar form of collagen I has received relatively little attention. This research focused on the ability of α2β1 integrin to act as a receptor for type I collagen fibrils. Also the molecular requirements of the EV1 interaction with α2β1 integrin were studied. Conventionally, ligand binding has been suggested to require integrin activation and the binding may further trigger integrin signaling. Another main objective of this study was to elucidate both the inside-out and outside-in signaling mechanisms of α2β1 integrin in adherent cells.

The results indicated that α2β1 integrin is the principal integrin-type collagen receptor for type I collagen fibrils, and α2β1 integrin may participate in the regulation of pericellular collagen fibrillogenesis. Furthermore, α2β1 integrin inside-out activation appeared to be synergistically regulated by integrin clustering and conformational activation. The triggering of α2β1 integrin outside-in signaling, however, was shown to require both conformational changes and clustering. In contrast to ECM ligands, EV1 appeared to take advantage of the bent, inactive form of α2β1 integrin in initiating its life cycle in the cell. This research together with other recent studies, has shed light on the molecular mechanisms of integrin activation. It is becoming evident that large ligands are able to bind to the bent form of integrin, which has been previously considered to be physiologically inactive. Consequently, our understanding of the conformational modulation of integrins upon activation is changing.

Key words: α2β1 integrin; collagen; echovirus 1; ligand binding; inside-out signaling; outside-in signaling

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α2β1 integriinin ligandin sitominen ja rakenteellinen säätely
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TIIVISTELMÄ

Integriinit ovat solukalvon läpäiseviä reseptoreita, joiden avulla solut kiinnittyvät ympäristöönsä. Integriinit osallistuvat myös useiden elintärkeiden biologisten tapahtumien säätelyyn solujen erilaistumisesta aina solukuolemaan asti. Useiden solutyyppien pinnalla esiintyvän α2β1 integriinin avulla solu kykenee tarttumaan erityisesti soluväliaineen säiemäiseen kollageeniin. Lisäksi echovirus 1 tunnistaa α2β1 integriinin tunkeutuakseen kohdesoluun. Tämän väitöskirjatyön tavoitteena oli selvittää α2β1 integriinin kahden ligandin, kollageenin ja echovirus 1:den, vuorovaikutusta rakenteellisesti eri muodoissa olevien α2β1 integriinien kanssa.

Vaikka soluväliaine koostuu pääosin kollageenisäikeistä, α2β1 integriinin vuorovaikutusta säiemuodossa olevan kollageenin kanssa oli työn alkaessa tutkittu vasta vähän. Tämän tutkimuksen yhtenä päätavoitteena oli selvittää solujen kykyä sitoutua α2β1 integriinin välityksellä säiemäiseen tyypin I kollageeniin. Lisäksi tavoitteena oli oppia ymmärtämään niitä α2β1 integriinin rakenteellisia yksityiskohtia, joita echovirus 1 hyödyntää päästäkseen soluun sisään. Tyypillisesti ligandin sitoutuminen integriiniin edellyttää ensin reseptorin aktivoitumisen, mikä puolestaan mahdollistaa solun sisäisen viestinvälitysketjun aloittamisen. Ligandin tunnistuksen lisäksi tutkimus pyrki selvittämään α2β1 integriinin aktivoitumisen ja viestinvälityksen vaatimia muutoksia reseptorin rakenteessa sekä sijainnissa solun pinnalla.

Tutkimustyön tulokset osoittivat α2β1 integriinin kykenevän toimimaan vuorovaikutuksessa säikeisen tyypin I kollageeniin kanssa. α2β1 integriinin todettiin jopa osallistuvan tyypin I kollageenin säiemuodostukseen. Integriinin aktivoitumisessa yksittäisen reseptorin rakenteellisten muutosten ja integriinien liikkumisen solukalvolla toistensa läheisyyteen osoitettiin vahvistavan toisiaan. Vaikka integriinin aktivoituminen itsessään ei vaatinut molempia tapoja, niiden todettiin olevan välttämättömiä solun sisäisen viestinvälitysketjun aloittamiseksi. Tutkimus osoitti lisäksi echoviruksen suosivan integriinin rakenteellista muotoa, jonka ei ole aiemmin havaittu kykenevän sitomaan isoja ligandeja. Yhdessä muiden tuoreiden tutkimusten kanssa väitöskirjatyön tulokset ovat muuttamassa käsitystä integriinien rakenteen ja toiminnan välisestä yhteydestä.

Avainsanat: α2β1 integriini; kollageeni; echovirus 1; ligandin sitominen; integriinin aktivaatio; viestinvälitys

TABLE OF CONTENTS

ABBREVIATIONS

ADMIDAS	adjacent to MIDAS
αI domain	inserted domain of the integrin alpha subunit
αMEM	alpha minimum essential medium
ATCC	American type culture collection
βI domain	beta inserted domain
βTD	beta tail domain
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DDR1, 2	discoidin domain receptor 1 and 2
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetate
EM	electron microscopy
EV	echovirus
FAK	focal adhesion kinase
FCS	fetal calf serum
GPIa/IIa	glycoprotein Ia/IIa
GST	glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline
HGF	human gingival fibroblast
I-EGF	integrin epidermal growth factor like domain
K_d	dissociation constant
kDa	kilodalton
MIDAS	metal ion-dependent adhesion site
p38 MAPK	protein 38 mitogen activated protein kinase
PBS	phosphate buffered saline
PKC	protein kinase C
PP2A	protein phosphatase 2A
TM	transmembrane
TPA	12-O-tetradecanoylphorbol-13-acetate
VLA	very late antigen
VP1-4	virus proteins 1-4
WT	wild type
vWF	von Willebrand factor

The single letter code for amino acids is used to depict important protein sequences. O refers to hydroxyproline.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I – III:

I. Connors, W.L., Jokinen, J., White, D.J., Puranen, J.S., Kankaanpää, P., Upla, P., Tulla, M., Johnson, M.S., and Heino, J. (2007). Two Synergistic Activation Mechanisms of α2β1 Integrin-Mediated Collagen Binding. *J.Biol.Chem.* **282**, 14675–14683.

II. Jokinen, J.*, Dadu, E.*, Nykvist, P., Käpylä, J., White, D.J, Ivaska, J., Vehviläinen, P., Reunanen, H., Larjava, H., Häkkinen, L., and Heino, J. (2004). Integrin-Mediated Cell Adhesion to Type I Collagen Fibrils. *J. Biol. Chem.* **279***,* 31956-31963.

III. Jokinen, J., White, D.J., Salmela, M., Huhtala, M., Käpylä, J., Sipilä, K., Puranen, J.S., Nissinen, L., Kankaanpää, P., Marjomäki, V., Hyypiä, T., Johnson, M.S., and Heino, J. (2010). Molecular mechanism of α2β1 integrin interaction with human echovirus 1. *EMBO J.* **29**, 196-208.

* These two authors contributed equally to this work.

1. INTRODUCTION

In order to form a dynamic functional unit individual cells in a multicellular organism need to communicate with their surroundings. Cell adhesion molecules, including integrins and other cell surface receptors, establish an important part of this communication. Integrins not only form a link between neighbouring cells, but also connect cells to the extracellular matrix (ECM), the material surrounding, supporting and binding the cells. Cell adhesion can be seen as a defining feature of multicellular organisms, and integrins are found even in the simplest metazoans, sponges and cnidarians (Burke 1999; Hughes 2001). Prokaryotes in turn, have integrin-type domains in their proteins (Whittaker and Hynes 2002).

In metazoans, integrin-mediated cell-cell and cell-extracellular matrix contacts lead to the activation of intracellular signaling pathways, which regulate a wide range of vital biological processes from development to tissue repair and homeostasis (Ruoslahti and Reed 1994; Hynes 1996). Out of the 24 distinct human integrin heterodimers, α2β1 integrin is a well-known receptor for collagens. The interaction of α2β1 integrin with collagenous matrix has been implicated in a number of biological and pathological conditions such as cancer, thrombosis, inflammation, angiogenesis and wound healing (Zutter and Santoro 2003). α2β1 integrin also serves as a cell surface receptor for some microbial pathogens including echovirus 1 (EV1). In humans, EV1 infection is associated with meningitis, encephalitis, rash, respiratory infections, diarrhea, and in some rare cases fatal neonatal illnesses (Grist *et al.* 1978).

This thesis describes the interaction of α2β1 integrin with its two high affinity ligands: EV1 and type I collagen in its fibrillar and monomeric forms. In addition to the ligand binding, the molecular mechanisms of α2β1 integrin inside-out and outside-in signaling were explored. The precise regulation of integrin activation is very important for their accurate function. The thorough understanding of the mechanisms of α2β1 integrin ligand binding and activation will provide insight into a cellular event of broad biological significance.

2. REVIEW OF THE LITERATURE

2.1. INTEGRIN FAMILY CONSISTS OF 24 MEMBERS

Integrins are heterodimeric transmembrane receptors composed of an α and a β subunit. The human genome encodes for 18 different α subunits and eight different β subunits. These form 24 distinct α-β combinations (Fig 1). Integrin α subunits can be divided into two different subgroups based on their structure: in humans, nine α subunits contain a ligand binding domain called the αI domain, while in the remaining 15 integrins the α and β subunits together form the ligand binding unit (Barczyk *et al.* 2010).

Integrin α subunits lacking the αI domain can be further divided into three groups based on their phylogeny (Johnson *et al.* 2009). Integrin α5, α8, αV and αIIb subunits bind to ECM components, which contain the arginine-glycine-aspartic acid (RGD) motif. This tripeptide is present in several extracellular matrix proteins, including fibronectin, vitronectin or fibrinogen (Ruoslahti and Pierschbacher 1987). Ancient RGD-binding integrins are found throughout the metazoa. A phylogenetically diverse second group includes the α3, α6 and α7 subunits, which recognize mainly laminin, the most important structural protein in basement membranes. The α4 and α9 subunits belong to a third group, and have evolved to recognize certain ECM proteins in addition to plasma proteins, immunoglobulins and vascular endothelial growth factors (Vlahakis *et al.* 2007; Johnson *et al.* 2009). These α subunits are known to recognize an acidic motif, leucine-aspartic acid-valine (LDV), which is functionally related to RGD. LDV is found in fibronectin, but also for example vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) contain related sequences (Humphries *et al.* 2006). This subdivision is based on the phylogeny of integrin α subunits, and it should be noted that the integrin α and β subunits have separate evolutionary origins (Johnson *et al.* 2009).

Although all metazoans have integrins, αI domain containing integrins appeared at a relatively late stage in evolution. Indeed, the integrin-type collagen receptors (α1β1, α2β1, α10β1 and α11β1) have only been found in vertebrates (Hughes 2001; Ewan *et al.* 2005; Huhtala *et al.* 2005). However, collagen itself arose at the very dawn of the metazoan world and is conserved in structure from the simplest sponges to complex vertebrates. In addition to collagen receptors, five αI domain containing integrin types (αLβ2, αMβ2, αXβ2, αDβ2 and αEβ7) are found in leucocytes, where they bind to counter-receptors on other cells or plasma proteins (Arnaout 1990; Springer 1990).

The existence of splice variants for some subunits further broadens integrin diversity. Two variants of the extracellular domain of the α6, α7, αIIb and β3 subunits have been reported (Bray *et al.* 1990; Djaffar *et al.* 1994; Delwel *et al.* 1995; Ziober *et al.* 1997; Schöber *et al.* 2000; Vijayan *et al.* 2000). It has been suggested that the differential splicing of the extracellular domains may modulate ligand binding specificity, and thus lead to the activation of diverging signaling pathways. Splice variants may also adopt different activation states or variation may affect heterodimerization (de Melker and Sonnenberg 1999). Correspondingly, the splicing of

the cytoplasmic domains ($\alpha3^{(A,B)}$, $\alpha6^{(A,B)}$, $\alpha7^{(A-C)}$, $\beta1^{(A-D)}$, $\beta3^{(A-C)}$, $\beta4^{(A-E)}$ and $\beta5^{(A,B)}$) may determine the integrin heterodimerization and affect integrin expression. Most importantly, variable cytoplasmic domains bind to different signaling proteins thus determining the cellular response. Like different integrin heterodimers, also splice variants seem to be tissue-specific and provide specialized ligand binding or signaling functions to the cell (Fornaro and Languino 1997; de Melker and Sonnenberg 1999; van der Flier and Sonnenberg 2001; Humphries *et al.* 2003).

The combination of the α and β subunits determines the ligand binding specificity of the integrin. However, many integrins recognize the same ligands. In the end, the combination of the integrin expression, an integrin activation pattern, and the availability of the ligand determines cell fate *in vivo*. For example, one single cell can express all four integrin-type collagen receptors simultaneously (Mirtti *et al.* 2006) implying that individual receptor types have separate functions.

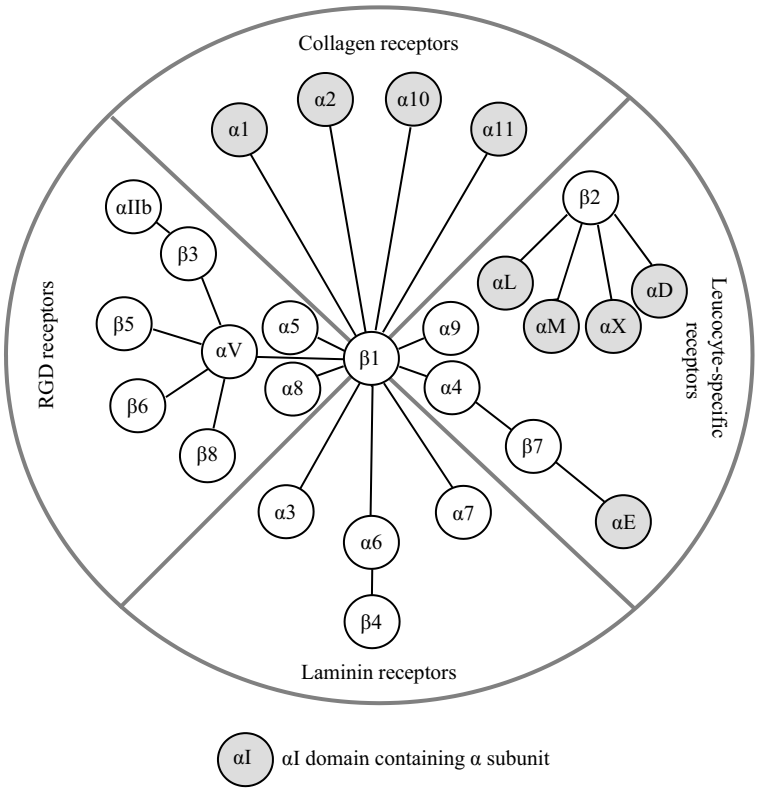


Figure 1. ***The integrin family of cell adhesion receptors consists of 24 α/β combinations.*** Nine integrin α subunits contain αI domain (●). αI domain containing integrins are classified as collagen receptors or leucocyte-specific receptors. αI domain lacking integrins (○) are further divided into three groups based on their phylogeny: RGD receptors, laminin receptors and LDV receptors. α4 and α9 containing integrins recognize LDV motif in their ligand and are classified as leucocyte-specific receptors in the figure. Figure modified from Barczyk *et al.* 2010.

2.1.1. Collagen receptor integrins

The ability of extracellular collagen to interact with cells was discovered much before its specific receptors were described. An early link between the α2β1 integrin and its function in platelet activation was obtained following the identification of a patient with defective α2β1 integrin expression on the surface of her platelets. As a consequence of the lack of the protein, the platelets were unresponsive to collagen and the patient suffered from prolonged posttraumatic bleeding (Nieuwenhuis *et al.* 1986). At the same time, α2β1 integrin was described by three different laboratories in different contexts. The receptor was identified as a very late activation antigen 2 (VLA-2) on the surface of T lymphocytes by Hemler and coworkers (1985), and as collagen receptor glycoprotein Ia/IIa (GPIa/IIa) by Santoro (1986). The large subunit of the collagen-binding extracellular matrix receptor II was described by Wayner and Carter (1987). The varying nomenclature reflects the important function and wide expression profile of α2β1 integrin. In 1987, Richard Hynes published a well-known scientific paper "Integrins: a Family of Cell Surface Receptors", where a consistent nomenclature for the whole integrin family, not only for collagen receptors, was used for the first time (Hynes 1987).

In addition to α2β1 integrin, other integrin-type collagen receptors are found in humans: α1β1 (VLA-1), α10β1 and α11β1. While α2β1 is widely expressed on the surface of platelets, fibroblasts, epithelial and endothelial cells (Zutter and Santoro 1990), α1β1 integrin is expressed in smooth muscle cells, fibroblasts, osteogenic cells, chondrocytes and lymphocytes. α10β1 and α11β1 represent the latest additions to the integrin family. α10β1 integrin was identified in human chondrocytes (Camper et al., 1998), while α11β1 was first identified as a major integrin on skeletal muscle cells and myotubes on cultured cells (Lehnert *et al.* 1999; Velling *et al.* 1999). However, later it has become apparent that muscle cells do not express the α11β1 protein *in vivo*. Instead, α11β1 integrin is found in the developing embryo where it is restricted to a subset of mesenchymal cells (Tiger *et al.* 2001; Popova *et al.* 2004).

Evolutionally, the α10β1 and α11β1 integrins are closer to each other than to the α1β1 or α2β1 integrins, which contain the most closely related integrin α chains (Velling *et al.* 1999). However, in ligand recognition the α1β1 integrin resembles α10β1 integrin while α2β1 and α11β1 integrins resemble each other. For example both α1β1 and α10β1 integrins bind basement membrane collagen IV with a higher affinity than they bind fibrillar collagen I, whereas α2β1 and α11β1 integrins display higher affinity towards collagen I than collagen IV (Kern *et al.* 1994; Tuckwell *et al.* 1995; Dickeson *et al.* 1999; Käpylä *et al.* 2000; Tiger *et al.* 2001; Tulla *et al.* 2001).

Despite their name "collagen receptor", α1β1, α2β1, α10β1 and α11β1 integrins also bind other important matrix molecules. Little is known about the ligand binding patterns of α10β1 and α11β1 integrins. However, α10β1 integrin has been reported to interact with laminin-111 (Tulla *et al.* 2001). Similarly α1β1 integrin recognizes not only laminin-111, but also laminin-112, laminin-511 (Colognato-Pyke *et al.* 1995; Colognato *et al.* 1997; Tulla *et al.* 2008) and cartilage protein matrilin-1 (Makihira *et al.* 1999). In contrast, α2β1 integrin has been connected with several matrix molecules. It is known to act as a receptor for laminin-111, -112 and -511 (Elices and Hemler

1989; Languino *et al.* 1989; Colognato *et al.* 1997; Tulla *et al.* 2008) as well as for tenascin C (Sriramarao, 1993), cartilage protein chondroadherin (Camper *et al.* 1997), decorin (Guidetti *et al.* 2002) and endorepellin, a C-terminal fragment of the heparin sulphate proteoglycan perlecan (Bix *et al.* 2004). Furthermore, matrix metalloprotease 1, which is involved in ECM breakdown, has been reported to be a ligand for α2β1 integrin (Dumin *et al.* 2001). In addition to extracellular matrix ligands, proteins such as C1q complement component and collectin family members, mannose-binding lectin and surfactant protein A bind α2β1 integrin highlighting the role of this integrin type in immunological responses (Zutter and Edelson 2007). Additionally, some viruses such as EV1 (Bergelson *et al.* 1992) and rotavirus SA11 (Hewish *et al.* 2000) have evolved to use an α2β1 integrin-mediated entry route. The following chapters will describe the ligand binding and consequent signaling mechanisms of α2β1 integrin.

2.2. FIBRILLAR COLLAGENS AND EV1 ARE LIGANDS FOR α2β1

2.2.1. Overview of the collagen family

The word collagen comes from Greek and describes the constituent of connective tissues that produce glue. Fibers in the connective tissues were first recognized in the 19th century. Later in the 1927, the French scientist Nageotte reported that acetic acid extracts from tissues could precipitate. This precipitate was later found to contain collagen fibers (reviewed in van der Rest and Garrone 1991). For the first decades after the discovery of type I collagen, it was the only collagen type to be known and studied. In the 1960s type II collagen was isolated from cartilage (Miller and Matukas 1969) and soon after type III collagen was found in fetal skin (Miller *et al.* 1971). Following the identification of the three fibril forming collagens, type IV collagen was found in basement membranes (Kefalides 1973). In the beginning, type IV collagen mystified researchers with its very different structural features compared to fibrillar collagen subtypes. Since then newly identified proteins have been categorized as collagens if they have the characteristic triple helical motif and if they are structural components of the ECM. However, the basic motif appears to be very adaptable to a range of functions and the latter definition excludes several proteins, such as adiponectin, acetylcholinesterase, collectins, complement component C1q, ectodysplasin, and ficolins from the collagen family (Myllyharju and Kivirikko 2004).

Collagen molecules are systematically numbered by Roman numerals in the order of their discovery. Currently, twenty-nine different collagen types are found in vertebrates. They are roughly categorized as fibrillar or nonfibrillar collagens. However, based on the structure and supramolecular organization of nonfibrillar collagens, they can be further divided into five subgroups: i) fibril-associated collagens with interrupted triple helices, ii) membrane-associated collagens with interrupted triple helices, iii) network-forming collagens, iv) collagens forming anchoring fibrils and v) collagens with multiple triple-helix domains and interruptions (multiplexins) (Shoulders and Raines 2009).

2.2.1.1. Characteristic repeats of Gly-X-Y dominate in collagen molecules

All proteins categorized as collagens contain three-stranded helical segments of a similar structure. The unique properties of each type of collagen are mainly due to segments that interrupt the triple helix and form characteristic folds. The common collagen structural unit has been known since the 1950´s and arises from an unusual abundance of three amino acids: glycine (Gly), proline and hydroxyproline (O). The characteristic sequence follows the amino acid pattern Gly-X-Y, where X is often proline and Y hydroxyproline. In human collagen strands, 22 % of all residues are either proline or hydroxyproline (Ramshaw *et al.* 1998). Glycine, the smallest amino acid without a side-chain, is required at every third position to accommodate close packing of the three twisting chains. In the assembly of the triple helix only glycine fits into the interior of the helix. To emphasize the importance of correct folding, many of the most damaging mutations to collagen genes result in the replacement of a glycine residue within the triple helix (Beck *et al.* 2000; Bodian *et al.* 2008). Proline stabilizes the rigid three-stranded collagen helix and the hydroxylation of proline by collagen prolyl 4-hydroxylases further increases the thermal stability of triple helices (Berg and Prockop 1973; Sakakibara *et al.* 1973; Myllyharju 2008). Each of the three polypeptide chains forms a left-handed helix. Together these three chains finally form a right-handed superhelix, named tropocollagen. Tropocollagen can be composed of three identical α-chains such as in type III fibrillar collagen, or the genes for their chains can be different; for example type I collagen is composed of two α1(I) chains and one α2(I) chain (Emanuel *et al.* 1985).

2.2.1.2. The subgroup of fibril forming collagens consists of seven collagen types

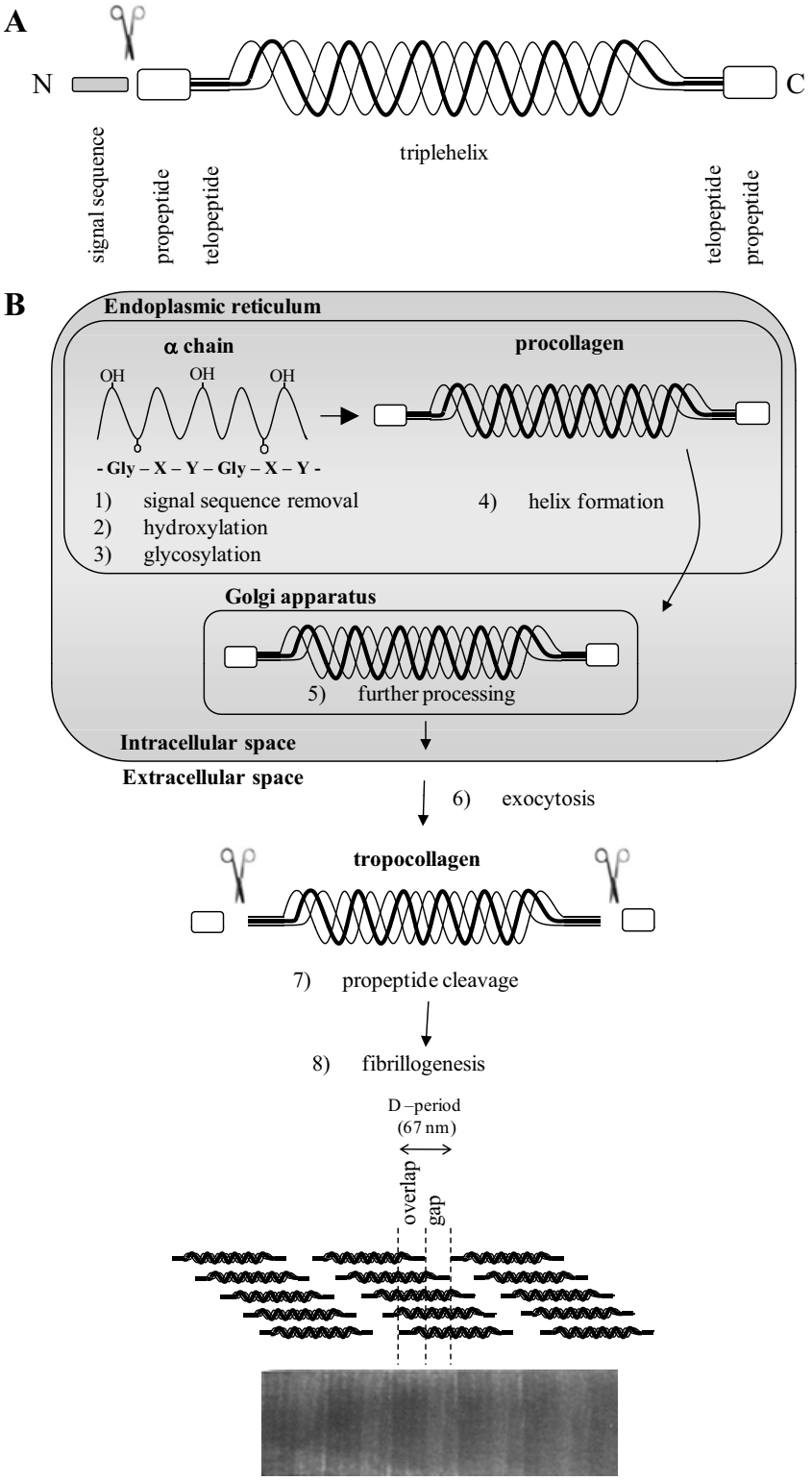
Type I collagen is the most abundant collagen type in the human body. It forms the predominant fibrils in dermis, ligaments, tendon, bone and cornea, while type II collagen fibrils are mostly found in hyaline cartilage. Type V and XI fibrillar collagens are often found in the same fibrils with type I and II, respectively (Wenstrup *et al.* 2006; Kadler *et al.* 2008). Type III collagen is present in skin, lung and the vascular system making it the second most abundant collagen in man (Boudko *et al.* 2008). The two most recent additions to the fibrillar collagen family, collagen types XXIV and XXVII, have unique molecular features: their triple helical sequences are shorter than those found in other fibrillar collagens. Additionally they have one to two interruptions in their sequence. Despite their exceptional structure, all other features classify collagens XXIV and XXVII as fibrillar collagens (Boot-Handford *et al.* 2003; Koch *et al.* 2003; Pace *et al.* 2003). Though the overall structure and banding pattern of collagen fibrils is very similar between tissues, fibrils have diverse physiological functions in different tissues. Consequently, the dimensions of fibrils and the degree of histological fibril organization varies (Myllyharju and Kivirikko 2001; Ottani *et al.* 2001). The next section will describe the basic mechanisms of type I collagen fibril formation.

2.2.1.3. From preprocollagen to collagen fibril

Collagen is synthesized as preprocollagen containing a short signal sequence at its N-terminus. In rough endoplasmic reticulum, the signal sequence is removed and triple-helical procollagen is formed (Fig 2A). In procollagen the triple-helical sequence is flanked by short telopeptides (11-26 amino acids). Next to the telopeptides there are a highly conserved non-collagenous sequence (250 amino acids) at the C-terminal end and a more variable (50-500 amino acids) propeptide at the N-terminal end (Fig 2A). Procollagens are first modified in the endoplasmic reticulum. At this point, some of their proline and lysine residues are hydroxylated (Fig 2B). These hydroxylations will stabilize the mature collagen by increasing the intra- and interstrand hydrogen bond formation. Additionally, lysyl hydroxylation enables the attachment of O-linked carbohydrates, such as galactose, to hydroxylysine, and further glucose to galactosylhydroxylysine. Lysine hydroxylation may also regulate the lateral packing of collagen molecules. Some asparagine and serine residues are targets for N-glycosylation and glycosaminoglycan attachment. From the endoplasmic reticulum the procollagens are transferred to the Golgi apparatus for further N-glycosylation. Finally, procollagen molecules are secreted into the extracellular space by exocytosis (Fig 2B; reviewed in Gordon and Hahn 2010).

Following secretion, the propeptides are cleaved off by specific procollagen metalloproteinases (Fig 2B). Mature tropocollagen, the monomeric collagen triple helix formed after the proteolysis of collagen propeptides, is approximately 300 nm long and 1.5 nm wide with about 1000 amino acids per chain. After removal of these globular domains, the solubility of collagen molecules decreases and they spontaneously start to form fibrils assisted by the remaining telopeptide domains. While the C-propeptides secure the collagen-type specific assembly and create a nucleation site for triple helix formation in a C to N direction, the uncleaved N-propeptides are supposed to limit further accretion of collagen molecules onto growing fibrils. Also telopeptides have an important function in maintaining fibril stability: neighbouring telopeptides interact with each other and are cross-linked covalently as a consequence of the action of lysyl oxidase (Orgel *et al.* 2000). Cross-links can be formed both within and between microfibers, and thus they help to maintain the twisted structure of the collagen microfibrils (Orgel *et al.* 2006; collagen biosynthesis is reviewed in Gordon and Hahn 2010).

Figure 2. ***Major events in the biosyntesis of collagen fibril.*** (A) Procollagen molecule consists of triple helical collagenous sequence flanked with pro– and telopeptides. The N –terminal signal sequence is removed in the endoplasmic reticulum. (B) In the endoplasmic reticulum and golgi apparatus, selected amino acids are modified by hydroxylation (OH) and glycosylation (○). Before the transportation from the endoplasmic reticulum to the golgi apparatus, three collagen α chains form a characteristic triple-helical structure, procollagen. Following exocytosis, propeptides are cleaved to form a mature collagen monomer, tropocollagen. Finally, tropocollagens are assembled head-to-tail and side-to-side forming D-periodic (67 nm) collagen fibrils.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OH
- Gly – X – Y – Gly – X – Y -
1) signal sequence removal
2) hydroxylation
3) glycosylation
4) helix formation
Golgi apparatus
5) further processing
Intracellular space
Extracellular space
6) exocytosis
tropocollagen
7) propeptide cleavage
8) fibrillogenesis
D –period
(67 nm)
overlap
gap

2.2.1.4. Collagen fibers are bundles of microfibrils

The most remarkable property of collagen fibrils is their regular banding pattern from tissue to tissue. In a simplified model, these D-periodic (Hodge and Petruska, 1963) stripes observed by electron microscopy (EM) arise when tropocollagens are aligned forming longitudinal gaps and axial overlaps. The length of the gap is 36.2 nm (0.54 D) and the overlap region of five adjacent molecules before the following gap is 30.8 nm (0.46 D), forming a D-period of 67 nm (Fig 2 B). Though the basic alignment of monomers is known, the arrangement of individual collagen molecules within the fibril has been a controversial issue for decades and numerous different models have been proposed (Hulmes and Miller 1979; Trus and Piez 1980; Hulmes *et al.* 1981; Bozec *et al.* 2007). At present, an arrangement of five collagen monomers into a quasi-hexagonal unit cell is the broadly accepted model (Orgel *et al.* 2001). Important details of the molecular packing within collagen fibrils were obtained from the first electron-density map of a type I collagen fiber (Orgel *et al.* 2006). The analysis supported the earlier observations that collagen monomers are arranged into supertwisted, right-handed microfibrils (Birk *et al.* 1989). These microfibrils were suggested to interdigitate, finally forming a mature collagen fiber with a spiral structure (Orgel *et al.* 2006). Atomic force microscopy studies support the model, and have described tendon collagen fibrils as "nanoscale ropes" because of the spiral disposition of the microfibrils (Bozec *et al.* 2007).

In vitro, collagen fibril formation is a self-assembly process, where external direction is not needed. In support of this, tropocollagen monomers have been reported to be unstable at body temperature. Thus, collagen fibrillogenesis has a stabilizing effect (Leikina *et al.* 2002). Despite the capacity to self-assemble *in vitro*, *in vivo* large collagen fibers are formed with the aid of several different classes of proteins such as other collagens, glycoproteins and proteoglycans (Hulmes 1992). The different structural requirements for example in tendon and cornea are met simply because of the alternating combinations of the modifying proteins in each tissue. While tendons require tensile strength, the essential feature of the cornea is transparency. Relatively small (~36 nm) and highly regulated collagen fibrils are needed to provide transparency to the cornea (Holmes *et al.* 2001; Meek and Fullwood 2001). To regulate the fibril diameter in cornea tissue, type V collagen integrates to type I collagen fibrils (Adachi and Hayashi 1986; White *et al.* 1997). Similarly, proteoglycans, such as lumicans (Chakravarti *et al.* 1998), decorin (Danielson *et al.* 1999) and fibromodulin (Svensson *et al.* 1999) have been reported to restrict fibril growth. Indeed, the notion of collagen self-assembly *in vivo* is overly exaggerated. Recently, it has even been shown that type I collagen fibril formation *in vivo* is not possible without the complex environment. Instead, the type I collagen fibrillogenesis has been shown to require at least fibronectin, collagen binding integrins and collagen V (Kadler *et al.* 2008).

2.2.2. General properties of EV1

Viruses have probably existed ever since living cells first evolved, and viruses are found wherever there is life (Iyer *et al.* 2006). The initial discoveries of the tobacco

mosaic virus by Dmitri Ivanovsky (1892) and Martinus Beijerinck (1898) was at the end of 19th century. Since 1899, when Friedrich Loeffler and Paul Frosch for the first time concluded that viruses could replicate and cause animal diseases, 5000 different strains, which is only a fraction of the estimated total amount of viruses, have been described in detail (reviewed in Lederberg 2000). The main motivation for the study of viruses has been the fact that they cause many harmful infectious diseases. Viruses can also be used as tools in molecular biology to study the function of several other proteins or to even cure diseases by using them in gene therapy. Also in the present study, EV1 was used as a tool to understand the structure-function relationship of collagen receptor α2β1 integrin.

Viruses are acellular organisms and their genetic information is encased within a protein capsule. To help them enter host cells, some viruses have an additional viral envelope covering their protein capsid. In order for a virus to replicate it must infect a permissive host cell. This feature explains the "name" virus which is Latin for *toxin* or *poison* and refers to the capability of the virus to kill the host cell (Lederberg 2000).

2.2.2.1. EV1 belongs to the family of Picornaviridae

Picornaviruses form a large group of the smallest known animal viruses. They cause infections such as Polio, Hepatitis A, and the common cold. Literally *pico***rna**virus means small RNA virus. Picornavirales consist of five different families: *Dicistroviridae, Iflaviridae, Marnaviridae, Picornaviridae and Secoviridae.* Echoviruses belong to the *Picornaviridae* family. This family is further separated into 12 different genera which cover 28 different species (Virus taxonomy: 2009 release, International Committee on Taxonomy of Viruses).

51 EV serotypes are categorized to the enterovirus genus, which consists of 10 species. EV1 is one of the serotypes placed into the Enterovirus B subgroup (Virus taxonomy: 2009 release, International Committee on Taxonomy of Viruses). The name echo is an acronym for "Enteric Cytopathic Human Orphan", where *orphan* designates a virus that was not originally associated with any clinically known disease. Nowadays, EV1 is connected with a variety of human diseases including diarrhea, rash, respiratory infections, meningitis and encephalitis (Grist *et al*. 1978).

2.2.2.2. The genetic material of EV1 is packed into the icosahedral capsid

The EV1 capsid encloses the viral genome. The particle consists of four viral proteins, VP1-4, is about 30 nm in diameter, and follows an icosahedral symmetry (Filman *et al.* 1998). The capsid proteins VP1-3 have two α-helices and a conserved eight-stranded β-barrel structural elements. The β-barrels are separated by highly variable loops (Hogle *et al.* 1985).

The icosahedral capsid consists of 60 protomers (Fig 3). Each protomer is composed of the structural proteins (VP1-3), which are located on the surface of the capsid, while a short capsid protein, VP4, is buried inside the protein shell (Filman *et*

al. 1998). Five protomers form a pentamer, and finally 12 pentamers comprise a viral capsid. The symmetric alignment of pentamers forms a depression or "canyon" around each fivefold symmetry vertex. These canyons can act as receptor recognition sites (Hogle *et al.* 1985; Rossmann *et al.* 2002).

The picornavirus genome consists of one single-stranded positive RNA molecule, which is 7.0–8.5 kilobases in length. The viral genome is translated from a single open reading frame in a cap-independent manner into one polyprotein that is then cleaved by virus encoded proteases into mature proteins. In addition to proteases and the structural capsid proteins, the viral RNA codes for other proteins involved in virus replication (Bedard and Semler 2004).

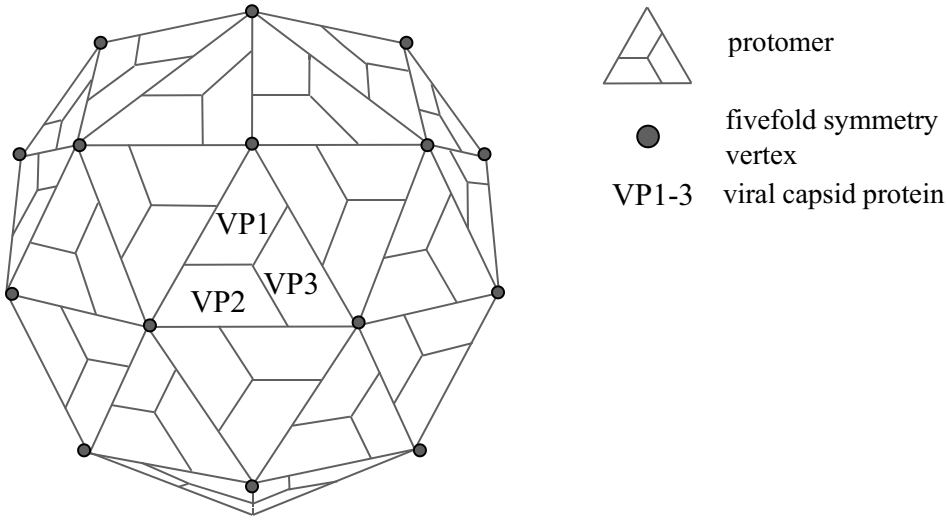

Figure 3. ***EV1 has an icosahedral capsid.*** EV1 capsid is composed of 60 copies of viral capsid proteins 1-4 (VP1-4). VP1-3 form the outer capsid shell and VP4 is buried inside the capsid. There is one copy of each protein in a protomer, and five protomers form a pentamer. The fivefold symmetry vertex of each pentamer is indicated in the figure (●).

2.2.2.3. The EV1 life cycle is dependent on the α2βl integrin

A first step in the viral life cycle is to recognize a binding molecule on the cell surface. All picornaviruses use type I transmembrane glycoproteins for entry into the cell. Among the cell surface receptors, numerous picornaviruses bind integrins and take advantage of their biology by promoting integrin signaling. For example coxsackievirus A9 uses αVβ1 and αVβ6 integrins to enter the cell (Chang *et al.* 1989; Roivainen *et al.* 1991; Roivainen *et al.* 1994; Williams *et al.* 2004), human parechovirus 1 takes advantage of αVβ1 and αVβ3 integrins (Hyypiä *et al.* 1992;

Stanway *et al.* 1994; Pulli *et al.* 1997; Joki-Korpela *et al.* 2001), while the foot-and-mouth disease virus entry is connected to αVβ3, αVβ6, α5β1 and αVβ1 integrins (Fox *et al.* 1989; Berinstein *et al.* 1995; Jackson *et al.* 1997; Jackson *et al.* 2000a; Jackson *et al.* 2000b). These viruses seem to mimic the extracellular matrix ligands of integrins by expressing the common integrin recognition sequence, RGD, on their surface. Recently, it was reported that some echoviruses, such as EV25, EV30, EV32, that do not have the RGD-motif, also use αVβ3 integrin to start their life cycle in the host cell (Ylipaasto *et al.* 2010). Similarly EV1 binds to α2β1 integrin in an RGD-independent manner (Bergelson et al., 1992).

Attachment to the α2I domain of the α2β1 integrin is the first important step in the EV1 life cycle (Fig 4; (Bergelson *et al.* 1992). Integrin-bound EV1 has been reported to cause α2β1 integrin clustering and to be internalized via tubulovesicular structures that quickly mature into pH neutral multivesicular bodies (Upla *et al.* 2004; Karjalainen *et al.* 2008). The pathway has been shown to be regulated by factors, which are associated with macropinocytosis such as protein kinase C, phospholipase C, phosphatidylinositol 3-kinase, Rac1, p21 activated kinase 1 and C-terminal binding protein 1 / brefeldin A-ADP ribosylated substrate (Ridley *et al.* 1992; Manser *et al.* 1994; Amyere *et al.* 2000; Grimmer *et al.* 2002; Upla *et al.* 2004; Karjalainen *et al.* 2008; Liberali *et al.* 2008). While EV1 has not been detected in any other cell organelles, such as the endoplasmic reticulum or the Golgi, virus uncoating and calpain-dependent replication may be initiated in these caveolin-positive structures (Pietiäinen *et al.* 2005; Upla *et al.* 2008).

EV1 uncoating has been reported to start 30 minutes post infection (Marjomäki *et al.* 2002). Decapsidated viral RNA can directly act as an mRNA and is translated into a polyprotein that is subsequently cleaved to form the mature viral proteins (Novak and Kirkegaard 1994). In EV1 infected cells, viral genomic RNA appears in the cytoplasm 2-3 h post infection (Pietiäinen *et al.* 2004) and newly synthesized capsid proteins and the viral RNA polymerase are detected 4 h post infection (Pietiäinen *et al.* 2004). Next, viral proteins form pentameric structures, which spontaneously assemble into viral capsids (Hogle 2002). Capsid formation is thought to be linked to the encapsidation of the RNA genome through a mechanism that is poorly understood (Nugent *et al.* 1999). Finally, cells are lysed and new infectious EV1 particles are released.

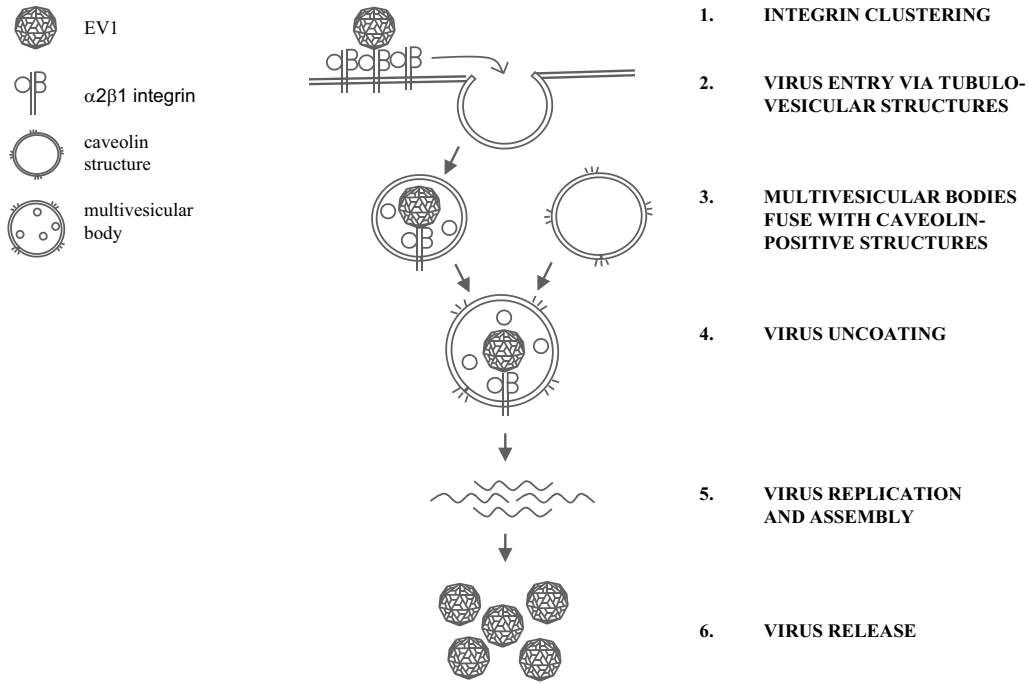

Figure 4. ***The model of the life cycle of EV1***. EV1 clusters α2β1 integrins and they are internalized together via tubulovesicular structures. These structures mature soon into multivesicular bodies. Next, multivesicular bodies fuse with caveolin –positive structures. In these vesicles EV1 has been proposed to start its uncoating. Following the virus replication in the cytoplasm, viral particles are assembled and the newly synthesized viruses are released from the cell by cell lysis.

2.3. LIGAND RECOGNITION OF α2β1 INTEGRIN

2.3.1. Overall structure of integrins

Integrins are heterodimeric transmembrane glycoproteins consisting of one α and one β subunit, which are non-covalently linked to each other. Both the α and β subunits have a large extracellular domain, single transmembrane helix and a short cytoplasmic tail. Only the β4 integrin forms an exception with its 1072 amino acid long cytoplasmic domain. Integrin extracellular parts are composed of similar structural domains. Thus, the information gained from the crystal structure of one integrin can be superimposed to other integrins in most regions. So far the crystal structures of five αI domains have been solved: αLI (Qu and Leahy 1995), αMI (Lee *et al.* 1995a; Lee *et al.* 1995b; Baldwin *et al.* 1998), α2I (Emsley *et al.* 1997), α1I (Nolte *et al.* 1999; Rich *et al.* 1999; Salminen *et al.* 1999; Nymalm *et al.* 2004) and

αXI (Vorup-Jensen *et al.* 2003). Additionally, for example α2I domain has been cocrystallized in complex with the collagen mimetic peptide glycine-phenylalanine-4-hydroxyproline-glycine-glutamate-arginine (GFOGER; (Emsley *et al.* 2000) and αLI with the inhibitor lovastatin and the ligand intercellular adhesion molecule-1 (Kallen *et al.* 1999; Shimaoka *et al.* 2003). Also 3D structures are available for α1I and α2I domains in complex with a specific amino acid sequence, arginine-lysine-lysine-histidine (RKKH), from the snake toxin metalloprotease jararhagin (Nymalm *et al.* 2004; Lambert *et al.* 2008). Furthermore, the crystal structures of the head domains and entire ectodomains including transmembrane parts have been determined for αVβ3 (Xiong *et al.* 2001; Xiong *et al.* 2009) and αIIbβ3 integrins (Xiao *et al.* 2004; Zhu *et al.* 2008). The αVβ3 integrin head domain has additionally been crystallized in complex with an RGD peptide (Xiong *et al.* 2002) and αIIbβ3 integrin with a ligand-mimetic antagonist (Xiao *et al.* 2004). Both the αV and αIIb subunits lack the αI domain, but recently also a crystal structure for the ectodomain of an αI domain containing integrin, αXβ2, has been solved (Xie *et al.* 2010).

The extracellular parts of the α and β subunits are composed of several structural domains (Fig 5). The larger αI domain containing α subunits (α2: 130 kDa) have five domains: an N-terminal seven-bladed β-propeller, where the αI domain is inserted between blades 2 and 3, an immunoglobulin-like Thigh domain and two large β-sandwich domains, Calf-1 and Calf-2. The integrin β-subunits (β1: 90 kDa) are formed by eight structural domains: an N-terminal βI inserted domain, which has been predicted to adopt a Rossmann fold similar to that found in the α subunit, an immunoglobulin-like "hybrid" domain, a cysteine-containing Plexin-Semaphorin-Integrin (PSI) domain reminiscent of that found in presenilins and semaphorins, four integrin epidermal growth factor-like domains (I-EGF) and a membrane proximal novel β tail domain (βTD). Together the extracellular parts of α and β subunits form the ligand-binding headpiece and tailpiece of the integrin (Fig 5).

Both integrin subunits span the cell membrane once. The hydrophobic transmembrane (TM) regions of both the α and β subunits are mostly composed of α-helixes. Two-helix bundles typically interact with each other via the motif glycine-xxx-glycine (GxxxG). This motif has been found in many TM proteins, for example glycophorin A (Popot and Engelman 2000). Integrins extend also into the cytosolic side of the membrane (Fig 5). Despite their small size (typically 15-66 amino acid residues) the cytoplasmic domains have a vital role in integrin function (Hynes 2002). Membrane-proximal regions contain a conserved glycine-phenylalanine-phenylalanine-lysine-arginine (GFFKR) motif in the α subunit and a leucine-leucine-xxx-histidine-aspartic acid-arginine-arginine-glutamic acid (LLxxxHDRRE) sequence is found in the β subunit (O'Toole *et al.* 1995; Calderwood 2004). These two membrane-proximal helices mediate the link between the subunits via a series of hydrophobic and electrostatic contacts. Additionally, the β-tail contains a critical asparagine-proline-x-tyrosine (NPxY) motif, which is required for receptor function (O'Toole et al 1995). Cytoplasmic regions that are not conserved may be critical for subunit specific integrin functions.

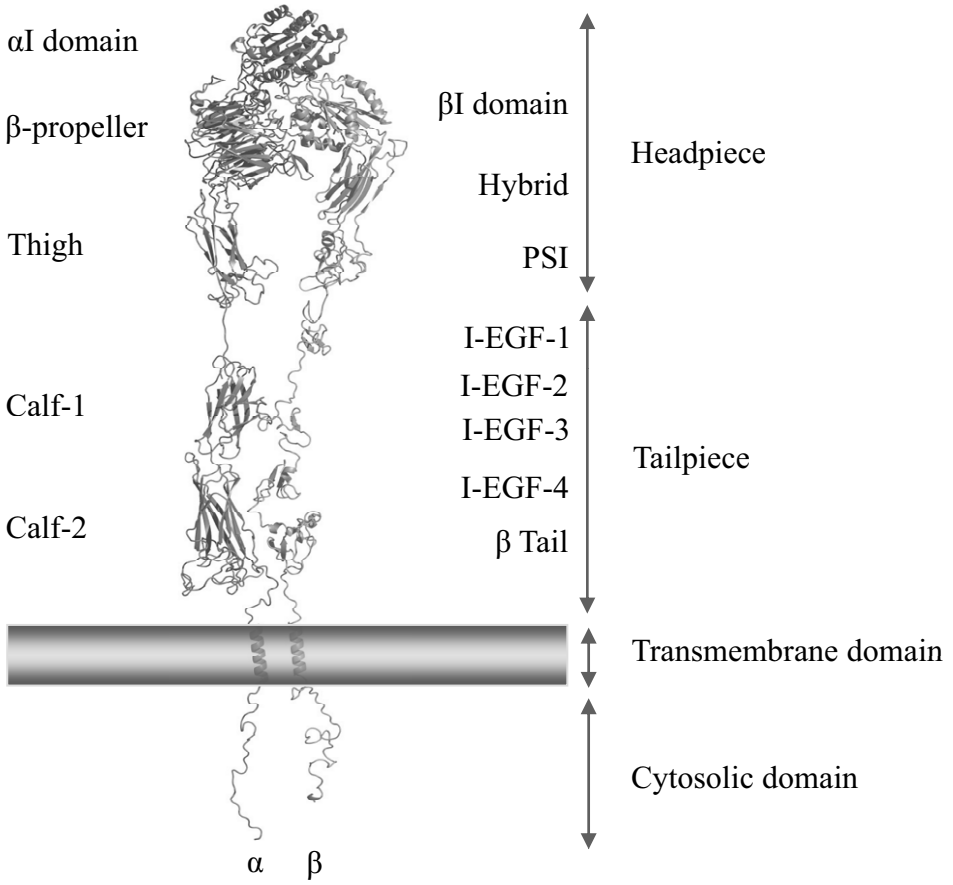

Figure 5. ***The structure of the integrin heterodimer.*** Both integrin α and β subunits are formed of extracellular, transmembrane and cytosolic domains. The extracellular domain can be further subdivided into headpiece and tailpiece. The extracellular portion of the α subunit consists of an αI domain, β-propeller, Thight and Calf domains 1 and 2. The corresponding part of the β subunit consists of βI domain, Hybrid domain, Plexin-Semaphorin-Integrin domain (PSI), four Integrin Epidermal Growth Factor like domains (I-EGF) and β tail domain. Structural modeling of α2β1 integrin by Mikko Huhtala.

2.3.2. The structure of α2I domain is similar with other αI domains

In some contexts, the integrin αI domain is also called the αA domain because of its structural similarity to Von Willebrand factor (vWF) A domains (Colombatti *et al.* 1993). vWF is a plasmaprotein involved in hemostasis. Its primary function is to bind other proteins, for example collagens (Pareti *et al.* 1986). Despite the structural similarity and collagen binding capacity, the ligand binding sites and motifs of the integrin αI domain and the vWF A domain differ from each other (Bienkowska *et al.* 1997; Emsley *et al.* 1997).

The integrin αI domain protrudes outwards between blades 2 and 3 of a seven-bladed β-propeller domain in the α subunit (Fig 6). Structurally the α2I domain, as well as other αI domains, adopts a classical dinucleotide binding fold, a Rossmann fold, with a core of five parallel (βA-E) and an anti-parallel (βF) β sheets surrounded by seven amphipathic α helices (Lee *et al.* 1995b). A conserved metal-binding site called the Metal Ion Dependent Adhesion Site (MIDAS), is located at the C-terminal end of the central β-sheet (Lee *et al.* 1995b). Next to the ligand binding MIDAS motif the α2I domain, as well as other collagen binding αI domains, has a unique extra α helix, the αC helix (Fig 6). The αC helix is a short turn-and-a-half α-helix and it creates a unique ligand binding groove for the αI domain in each collagen binding integrin (Emsley *et al.* 2000; Käpylä *et al.* 2000).

When amino acid sequences from human, cow, mouse and pig are compared, only 43 non-conserved residues are found in the α2I domain. As an indication of indispensable sites for ligand binding, the MIDAS face and also the αC helix are always invariant. Indeed, the consensus sequence aspartic acid-x-serine-x-serine (DxSxS) motif with an additional threonine, which coordinates metal ion, Mg^{2+} or Mn^{2+}, is conserved among α2I domains, and mutation in any of these residues abrogates ligand binding. Interestingly, 41 of the variant residues lie on the surface of the α2I, in the residues surrounding the MIDAS (Emsley *et al.* 1997). These amino acids have been found to be important in the selective recognition of ligands (Michishita *et al.* 1993; Kamata *et al.* 1994; Kern *et al.* 1994; Edwards *et al.* 1995; Huang and Springer 1995; Kamata *et al.* 1999; Emsley *et al.* 2000; Smith *et al.* 2000).

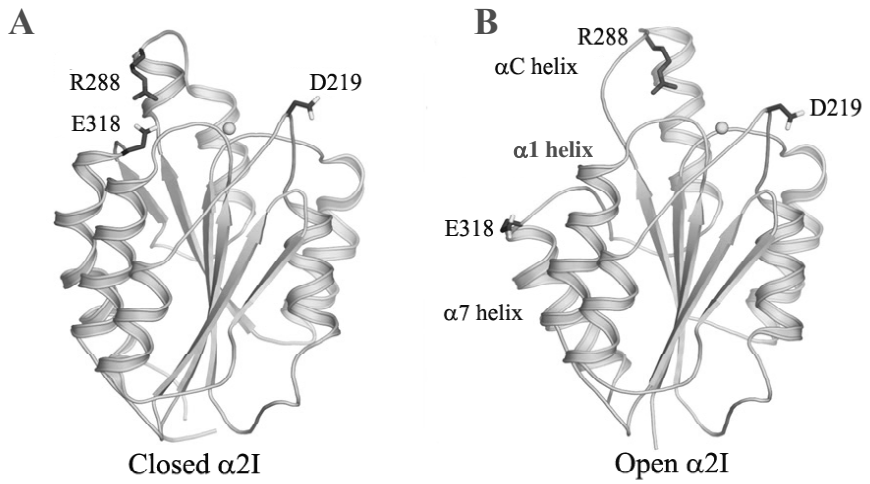

Figure 6. ***The structure of the α2I domain.*** α2I domain adopts a Rossman fold with a core of five parallel and one anti-parallel β sheets (β sheet structures depicted as arrows in the figure) surrounded by seven amphipathic α helices. MIDAS coordinated divalent metal ion is indicated with a sphere in the figure. In a closed α2I domain conformation (A) residues R288 and E318 form a salt bridge. The disruption of this salt bridge leads to the open α2I domain conformation (B), where the locations of αC, α1 and α7 helices are changed. Residue D219 is an important residue in determining α2I domain ligand binding specificity. Modified picture from Tulla *et al.* 2008.

2.3.3. Small differences define the ligand binding patterns of α1I and α2I

α1I and α2I domains have a high sequence identity, yet, the ligand binding patterns of the two domains are different. Despite the overall similarity in ligand binding properties, α2I domain has been reported to bind to collagen in a slightly different manner compared to α1I domain (Xu *et al.* 2000). A comparison of the structures of α1I and α2I domains revealed that the αC helix in α2I domain is more protruding than in α1I domain, contributing to the smaller dimensions of its trench in α2I domain (Rich *et al.* 1999). The αC helix of α2I domain is located in a ligand binding area and was thus first suggested to have a critical role in ligand binding. However, the crystal structure of α2I domain in complex with GFOGER, indicates that the αC helix is not in a direct contact with a collagenous ligand. The location of the αC helix in the α2I domain, however, has been shown to change during conformational modulation (Fig 6), and the possibility that the αC helix would act as a steric barrier for non-specific collagen binding has not been excluded (Emsley *et al.* 2000; Emsley *et al.* 2004).

When the crystal structure of a ligand bound α2I domain was further carefully examined, one negatively charged amino acid, aspartic acid 219 (D219, see Fig 6), was found to form a contact with the collagenous peptide (Emsley *et al.* 2000). The corresponding amino acid in α1I domain, arginine 218 (R218), is positively charged. Mutational studies have confirmed that among other small factors these amino acids with different charges are important in determining ligand binding selectivity (Tulla *et al.* 2001).

2.3.4. Ligand binding induces conformational change in the α2I domain

Integrin αI domains are thought to exist in two conformations: open and closed (Lee *et al.* 1995a; Lee *et al.* 1995b; Emsley *et al.* 2000). There are differences in the metal ion coordination in the MIDAS between the two conformations. In a closed conformation the metal ion is coordinated by the aspartic acid from the αI domain. In the open structure, an acidic residue from an exogenous ligand can occupy the MIDAS and provide the sixth coordination site for the bound metal ion (Emsley *et al.* 2000; Arnaout *et al.* 2005). When the α2I domain conformation changes from closed to open (Fig 6), the modulation in the metal ion coordination leads to further conformational changes: the N-terminal α1 helix of the α2I domain moves inward, the αC helix unwinds, and finally the C-terminal α7 helix moves downward (Emsley *et al.* 2000; Arnaout *et al.* 2005). The formed open α2I conformation has been suggested to have a high affinity to ligand, while the closed α2I domain conformation is associated with the low affinity to ligand. To support this hypothesis, constitutively open α2I domains have been reported to have a high affinity to their ligands (Aquilina *et al.* 2002; Tulla *et al.* 2008). Furthermore, the region undergoing the extensive conformational changes has been found to be accessible for an antibody, which is suggested to recognize only the active integrin conformation (Oxvig *et al.* 1999). While the ligand binding itself triggers the changes in the tertiary structure of the α2I domain, it has been proposed that the conformational changes are not required for the initial ligand recognition (Siljander *et al.* 2004).

2.3.5. The mechanism of α2β1 integrin binding to collagen

The triple helical sequence of fibril forming collagens that fulfills the coordination of the metal ion, is contained entirely within the six-residue sequence GFOGER (Knight *et al.* 1998; Emsley *et al.* 2000; Knight *et al.* 2000; Xu *et al.* 2000; Zhang *et al.* 2003). While this hexapeptide is required for a proper triple helical conformation, α2I domain interacts mainly with one α chain of collagen (Morton *et al.* 1997; Emsley *et al.* 2000). In the collagen binding motif the acidic residue glutamate (E) coordinates the metal ion. As a reflection of the importance of the glutamate, replacing it with aspartate eliminates the integrin binding (Knight *et al.* 2000). Presumably aspartate is too short to reach the metal ion. However, not only glutamate but also other residues in the hexapeptide are important: arginine (R) forms a salt bridge to an aspartate residue in α2I domain, while phenylalanine (F) makes hydrophobic contacts with α2I domain. In addition to GFOGER, there are also other α2β1 integrin binding motifs on collagen, such as the glycine-leucine-4-hydroxyproline-glycine-glutamate-arginine (GLOGER), glycine-methionine-4-hydroxyproline-glycine-glutamate-arginine (GMOGER), glycine-alanine-serine-glycine-glutamate-arginine (GASGER), glycine-arginine- 4-hydroxyproline-glycine-glutamate-arginine (GROGER) and glycine-alanine-4-hydroxyproline-glycine-glutamate-arginine (GAOGER; Xu *et al.* 2000; Kim *et al.* 2005; Raynal *et al.* 2006). The open conformation of the αI domain has been reported to further increase the variability of the GxxGER motifs, that are recognized by the α2β1 integrin (Siljander *et al.* 2004). It is also noteworthy that different collagens represent different GxxGER type integrin binding sites, and in addition to this, alternative binding motifs and mechanisms exist. For example, α1β1 integrin recognizes three amino acid residues, arginine and two aspartic acids, located in three separate chains of the type IV collagen helix (Golbik *et al.* 2000).

2.3.6. The mechanism of EV1 binding to α2β1 integrin

Both collagen and EV1 bind to overlapping sites on the α2I domain. However, the binding mechanisms of these two ligands are evidently different. In contrast to collagen binding, the attachment of EV1 is independent of divalent cations (Bergelson *et al.* 1993). Due to the overlapping binding sites, the α2I domain cannot bind both collagen and EV1 simultaneously (King *et al.* 1997; Xing *et al.* 2004). Therefore, EV1 must compete with native ligands for free α2β1 receptors. Viruses have evolved to partially solve this problem with their capacity to bind to the receptor tighter than any of the natural α2β1 integrin ligands (Xing *et al.* 2004).

In a cryoEM analysis, the virus particle appeared to be decorated with 60 copies of the α2I domain suggesting that each protomer formed of the viral capsid proteins VP1-3, could occupy one α2I domain. Accordingly, α2I domain was shown to interact with the VP2 of one protomer and the VP3 of a neighboring protomer in the EV1 capsid (Fig 7). Charged residues in EV1 were found to form electrostatic interactions with the α2I domain (Xing *et al.* 2004). In the α2I domain the only existing charged residues in the EV1 binding interface, Lys201, Asp219 and Arg288, have been suggested to participate in the EV1 binding (Xing *et al.* 2004). To support the cryoEM

reconstruction, mutational studies have suggested that two groups of amino acid residues, 199-201 and 212-216, in α2I as well as Asp289 are involved in virus attachment (King *et al.* 1997; Dickeson *et al.* 1999). None of those critical α2I residues indicated by mutational studies are located in the MIDAS motif. Instead they all lie on a fairly flat surface on one face of the α2I domain in a site that has been suggested to be easily accessible to viruses. While the cryo-EM model described only the binding of the α2I domain to the virus capsid protomer, there seems to be no steric hindrance, which would inhibit a viral pentamer in occupying five entire α2β1 integrins (Xing *et al.* 2004). The model of closed α2I binding to the surface of EV1 is shown in figure 7.

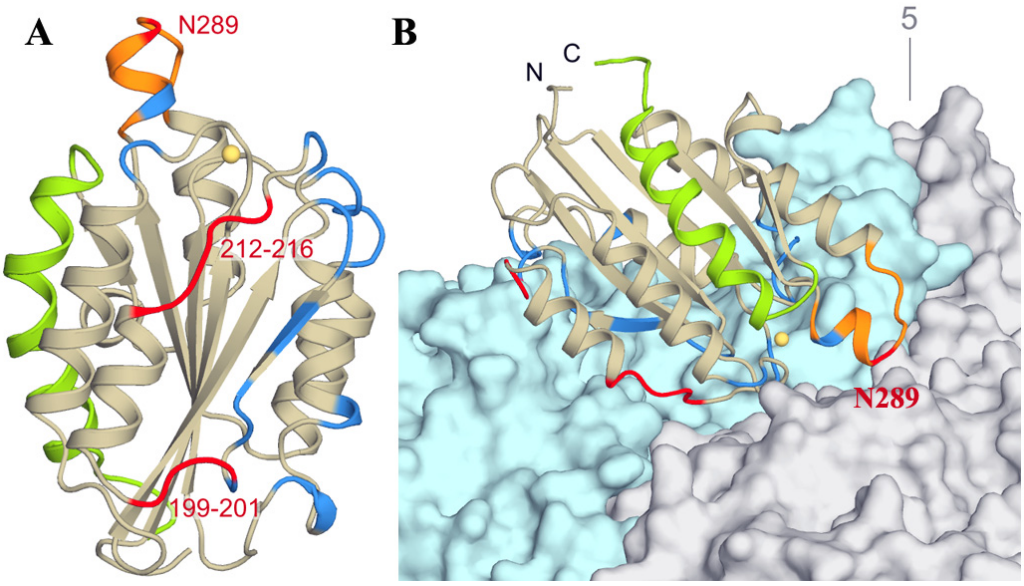

Figure 7. ***The model of EV1 binding to α2I.*** The closed α2I domain (A) is docked onto the surface of EV1 capsid (B). Two protomers of EV1 are shown; one is colored grey and the other one light blue. The five fold symmetry vertex is marked `5`. The residues that have been shown by mutagenesis to affect EV1 binding are coloured red (residues 199-201, 212-216 and 289). Modified picture from publication III.

2.4. α2β1 INTEGRIN SIGNALING AND FUNCTION

Integrins can mediate bidirectional signals across the cell membrane, and their activation is regulated both temporally and spatially. A good example of the importance of integrin regulation comes from circulating platelets, in which incorrect integrin activation can lead to pathological conditions, such as thrombosis. Indeed, many integrins are expressed on the surface of platelets in an inactive state with reduced capacity for ligand binding and signaling (Hynes 2002). Inside-out signaling activates integrins, while outside-in signaling triggered by integrin ligand binding can regulate a broad array of cellular processes such as cell survival, differentiation, proliferation, and migration.

2.4.1. Inside-out signaling primes integrins for ligand binding

Integrin ligand binding is modulated by low and high affinity conformations. The conformational shift is elicited by signals that induce intracellular regulatory proteins to bind to the cytoplasmic domains of integrins to initiate integrin inside-out signaling (Lu *et al.* 2001a; Kim *et al.* 2003; Kim *et al.* 2004). One of these cellular activators is talin. Talin is a 250 kDa cytoskeletal protein that is composed of a 47 kDa N-terminal head domain and a 190 kDa C-terminal rod domain (Calderwood *et al.* 1999; Calderwood *et al.* 2002; Vinogradova *et al.* 2002). The activated talin head domain has been shown to bind directly to the integrin β cytoplasmic tail at both the NPxY – containing region and the membrane-proximal helix (Liu *et al.* 2000). Talin binding, and the consequent upward movement of the β1 tail into the cell membrane, induce the dissociation of the clasp between the integrin α and β subunits (O'Toole *et al.* 1991; O'Toole *et al.* 1994; Hughes *et al.* 1996; Lu *et al.* 2001b; Takagi *et al.* 2001; Vinogradova *et al.* 2002; Kim *et al.* 2003; Vinogradova *et al.* 2004). While talin alone has been shown to be sufficient for integrin activation (Ye *et al.* 2010), there is plenty of recent evidence that strongly stresses the role of kindlins in integrin activation (Ma *et al.* 2008; Montanez *et al.* 2008; Moser *et al.* 2008; Ussar *et al.* 2008; Moser *et al.* 2009). Kindlin and talin have been suggested to cooperate. However, the exact mechanism of kindlin action is still to be uncovered. Finally, the binding of regulatory proteins to the integrin cytosolic tails have been reported to induce the separation of the cytosolic and transmembrane domains of the integrin α and β subunits (O'Toole *et al.* 1991; O'Toole *et al.* 1994; Hughes *et al.* 1995; Hughes *et al.* 1996; Gottschalk 2005; Czuchra *et al.* 2006). This change has been proposed to further induce conformational modulation in the extracellular domains of integrins. However, the exact mechanism of the shift from the low affinity to the high affinity conformation is a matter of controversy.

There are two predominant models that predict the conformational changes required to facilitate integrin ligand binding: the "switchblade" (Beglova *et al.* 2002; Shimaoka *et al.* 2002; Takagi *et al.* 2002; Nishida *et al.* 2006; Luo *et al.* 2007) and the "deadbolt" models (Xiong *et al.* 2003; Arnaout *et al.* 2005). In the "switchblade" model the ligand binding affinity has been suggested to increase when the integrin shifts from the bent conformation to the extended conformation (Fig 8A-B). The "deadbolt" model in turn proposes that also the bent integrin conformation is able to bind the ligand (Fig 8A). Regardless of the suggested conformation of the high-affinity integrin both models are in agreement with the idea that the integrin head domain, comprised of the βI domain of the β subunit and the β-propeller and the αI domain of the α subunit, goes through the conformational modulation during activation.

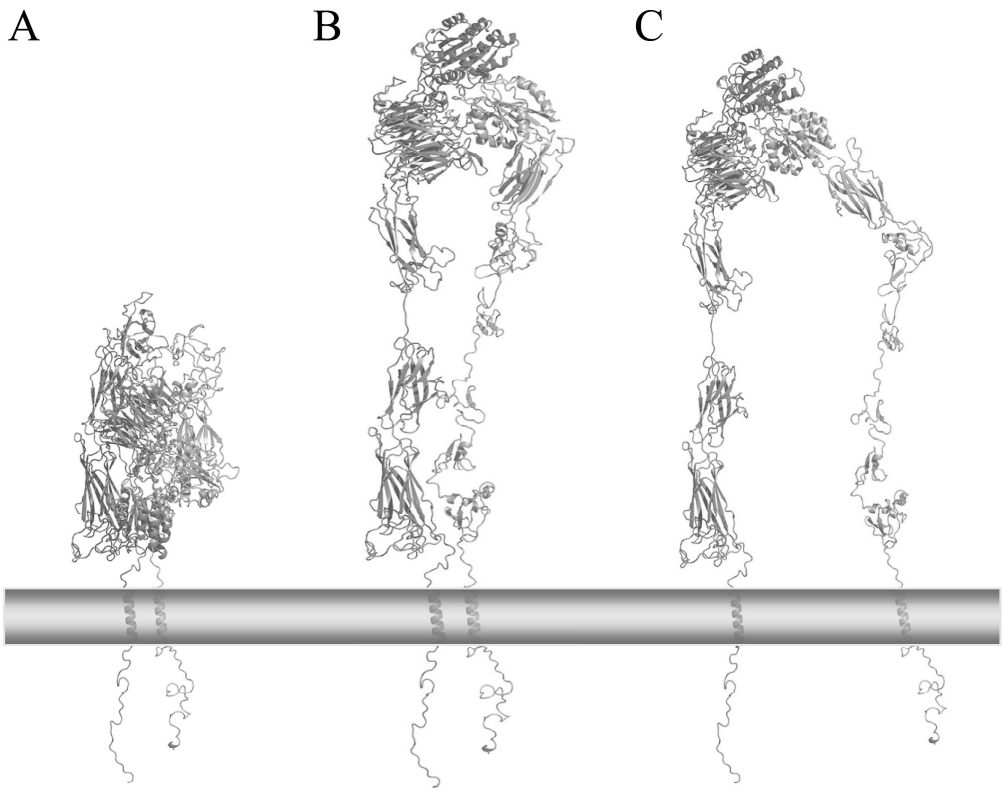

Figure 8. ***Integrin activation.*** According to a conventional model of integrin activation, integrins adopt two predominant conformations: a bent (A) and an extended (B) one. Ligand binding has been suggested to lead to the separation of the transmembrane and cytosolic domains of the integrin α and β subunits (C). Structural modeling of α2β1 integrins by Mikko Huhtala.

In a bent conformation, the integrin head region is closely juxtaposed with the cell membrane (Fig 8A). Ligand binding has been proposed to be unfavorable, when the ligand binding region is oriented toward the plasma membrane. In the "switchblade" model the extended conformation of the integrin (Fig 8B) is suggested to be induced through changes in several structural domains. First the separations of the cytoplasmic and transmembrane domains lead to the outward swing of the hybrid domain in the β subunit relative to the βI domain. This shift is driven by a downward slide of the α7 helix in the C-terminal βI domain. The slide causes changes in cation coordination in the βI MIDAS, so that the βI domain is finally able to interact with the β-propeller in the α-subunit. To support the "switchblade" model, both an αI domain lacking αVβ3 integrin and an αI domain containing αXβ2 integrin have been proposed to adopt a bent conformation in the crystal structures of ectodomains in the absence of a ligand (Xie *et al.* 2010). Furthermore, these crystal structures are in agreement with other studies using EM (Takagi *et al.* 2002; Takagi *et al.* 2003; Iwasaki *et al.* 2005; Nishida *et al.* 2006), nuclear magnetic resonance technique (Beglova *et al.* 2002) as well as mutational analyses (Luo *et al.* 2004; Tng *et al.* 2004; Mould *et al.* 2005; Tang *et al.* 2005).

The "deadbolt" model proposes that integrin affinity for a ligand is regulated across a single domain without an extended structure. The model is based on the crystal structures of αVβ3 integrin without the ligand and in complex with the allosteric inhibitor lovastatin or fibronectin (Xiong *et al.* 2001; Xiong *et al.* 2002; Adair *et al.* 2005). In the "deadbolt" model, a flexible loop in the βTD domain in the leg region of the β3 subunit has been suggested to act as a deadbolt and lock the βI domain in the low affinity state. A hairpin loop from βTD has been predicted to interact with βI domain. This deadbolt has been proposed to prevent the coordination of the metal ion in the β1 MIDAS leading to a low affinity towards the ligand. This interaction has been suggested to be reversibly disengaged by integrin inside-out signaling (Masumoto and Hemler 1993; Hughes *et al.* 1996; Xiong *et al.* 2003). The model perceives a rapid and reversible activation required for precise integrin regulation (Xiong *et al.* 2003). It also aims to explain the findings implying that the bent integrin conformation is able to bind a small ligand (Adair *et al.* 2005) or that the extended integrin conformation does not necessarily present the active high affinity conformation (Takagi *et al.* 2001).

2.4.2. Ligand binding induces integrin outside-in signaling

Activated integrins are able to bind a ligand and initiate integrin outside-in signaling. The crystal structure of the αVβ3 integrin in a complex with an RGD peptide reveals a link between the ligand binding and the quaternary structural change in the αI domain free integrin (Xiong *et al.* 2002). It appears that Asp (D) from the RGD coordinates the metal ion in the MIDAS of the βI domain. Following ligand binding, two loops from βI, one from the MIDAS, form a link between the β-subunit and the β-propeller domain in the α subunit. Thus, ligand binding causes large changes in the quaternary structure of the integrin (Xiong *et al.* 2002). In the αI domain containing integrins, a conserved Glu residue in the αI domain is thought to act as an intrinsic ligand for the βI domain and cause structural changes similar to those

triggered by the direct binding of the Asp of the RGD ligand to βI domain (Liddington and Ginsberg 2002). Ligand binding to the αI domain first triggers the recoordination of the metal ion in the MIDAS as previously discussed. These rearrangements also lead to structural changes in the bottom face of the αI domain, especially in the α7 helix. An invariant Glu in αI domains is the first residue in the α7 helix in the αI C-linker region. Ligand binding causes downward movement of the α7 helix, so that it is able to become in contact with the βI domain. Recent studies, however, reveal that the αI domain is more flexible than was previously suggested, and instead of the helix movement, tilting of αI domain alone would lead to the engagement of the αI Glu residue with the βI MIDAS (Shimaoka *et al.* 2002; Xie *et al.* 2010). Finally, the conformational change in integrin head domain is transmitted to cytosolic domains, which become separated allowing signaling proteins to bind and focal adhesions to form (Fig 8C).

2.4.3. Formation of focal adhesions

A conformational change within a single receptor molecule increases the affinity of an integrin to its ligand. To strengthen integrin-mediated cell adhesion, receptors move laterally onto a certain spot on the cell membrane forming integrin clusters, thus increasing integrin valency (Faull *et al.* 1994; Loftus *et al.* 1994; Stewart and Hogg 1996; Bazzoni and Hemler 1998; van Kooyk *et al.* 1999). Integrin clustering forms dynamic focal adhesion sites, where cytoskeletal proteins are recruited to transmit both integrin-mediated mechanical forces and regulatory signals between cells and the ECM.

The binding of talin head domain to the β subunit switches the integrin into active state. However, without the talin rod domain, talin is not able to link the integrin to the cytoskeleton (Tanentzapf and Brown 2006; Moes *et al.* 2007). In early focal complexes, the adapter protein paxillin may link integrin to the cytoskeleton (Alon *et al.* 2005). Furthermore, other proteins such as the actin binding vinculin and the actin bundling homodimer α-actinin are incorporated into the complex strengthening contacts with cytoskeleton (Laukaitis *et al.* 2001; Zaidel-Bar *et al.* 2003; Kelly and Taylor 2005). In addition to typical proteins that form a link between integrin and cytoskeleton, also focal adhesion tyrosine kinase (FAK) plays an important role in the signaling networks at focal contacts (Mitra *et al.* 2005). The activation of common regulatory and signaling proteins, such as talin or FAK, is typical of several, if not all, integrins (Parsons *et al.* 2000). However, integrins also induce heterodimer specific signaling. That is because each integrin forms specific interactions with cellular components using the non-homologous parts of the cytoplasmic domain of the α subunit. The distinct signaling properties partially rationalize why one single cell can express for example all four collagen receptor integrins simultaneously.

The response of each cell is always determined by a unique combination of integrins and other signaling receptors on the cell surface, in addition to the signaling microenvironment of the cytosol. Thus, the cell's fate is the result of the interplay between many other cell membrane receptors and signaling proteins of the cell. To further intricate integrin-mediated cell signaling, the α2β1 integrin action seems to be

cell type specific and several signaling proteins recruited by α2β1 integrin have been described. α2β1 activates different kinases, such as mitogen activated protein kinase p38 (p38 MAPK; (Ivaska *et al.* 1999b), Akt/protein kinase B and phosphatidylinositol 3-kinase (Ivaska *et al.* 2002). In addition to kinases, α2β1 integrin also activates protein phosphatase 2A (PP2A; (Ivaska *et al.* 2002), a cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Koyama *et al.* 1996; Henriet *et al.* 2000), osteoblast specific transcription factor- 2 (Xiao *et al.* 1998), and a member of the Rho family GTPases, Rac-1, and its guanine nucleotide exchange factor Vav-2 (Arora *et al.* 2008).

2.4.4. Cell proliferation and survival are dependent on the organization of ECM *in vitro*

Most of the matrix binding integrins form similar focal adhesion sites on two-dimensional monolayers. However, in the three-dimensional matrix the formation of focal adhesions, and consequently cell signaling events might be somewhat different (Henriet *et al.* 2000; Cukierman *et al.* 2001). For example, it has been shown in different cell types that α2β1 integrin mediated cell adhesion to three-dimensional fibrillar collagen matrix inhibits the cell proliferation, while cell adhesion to two-dimensional matrix induces the process (Koyama *et al.* 1996; Fluck *et al.* 1998; Henriet *et al.* 2000).

In melanoma cells and smooth muscle cells, α2β1 integrin appears to affect specific proteins essential for cell cycle progression such as $p27^{KIP1}$. α2β1 integrin binding to three-dimensional fibrillar type I collagen has been shown to upregulate $p27^{KIP1}$ leading to cell cycle arrest (Koyama *et al.* 1996; Henriet *et al.* 2000). In contrast, on two-dimensional monomeric collagen $p27^{KIP1}$ is downregulated and α2β1 integrin appears to increase cell proliferation (Zutter *et al.* 1999). The mechanism of $p27^{KIP1}$ function in this context is still unclear. However, it has been speculated that α2β1 integrins would not be able to cluster when bound to collagen fibrils and this would cause a cell cycle arrest (Henriet *et al.* 2000). In addition to $p27^{KIP1}$, α2β1 integrin activates PP2A in fibroblasts and osteosarcoma cells. PP2A activation can be an alternative pathway for the cell to escape from the cell cycle. Activation of PP2A might also lead to dephosphorylation of Akt/protein kinase B, which is a promoter for cell survival (Ivaska *et al.* 2002). Indeed, α2β1 integrin has been shown to protect cells from apoptosis (Saelman *et al.* 1995; Aoudjit and Vuori 2000; Baeckström *et al.* 2000; Smida Rezgui *et al.* 2000).

2.4.5. α2β1 integrin induces cell invasion and migration *in vitro*

Mechanistically cell invasion and migration are complex processes, which require matrix remodeling, and the timely attachment and detachment of cells to and from the ECM. In several cell lines collagen-induced p38 MAPK activation is strongly linked to α2β1 integrin (Ivaska *et al.* 1999b; Ravanti *et al.* 1999; Klekotka *et al.* 2001b; Xu *et al.* 2001) and p38 MAPK signaling may in turn lead to a migratory cell phenotype (Klekotka *et al.* 2001a; Klekotka *et al.* 2001b). In the dynamic extracellular matrix remodeling, the regulation of collagen synthesis and degradation is important. Over-

expression of α2β1 integrin has been reported to increase collagen mRNA synthesis *in vitro* by a mechanism which involves p38 MAPK activation (Riikonen *et al.* 1995; Ivaska *et al.* 1999b). While α2β1 integrin induces collagen synthesis by stimulating the p38 MAPK pathway, the kinase is also responsible for the upregulation of collagenase-3 (matrix metalloproteinase-13; Ravanti *et al.* 1999). Furthermore, cell contacts with three dimensional collagen are known to activate the expression of collagenase-1 (matrix metalloproteinase-1) in an α2β1 integrin-dependent manner (Langholz *et al.* 1995; Riikonen *et al.* 1995). The *in vitro* results described above suggest that α2β1 integrin may be involved in both cell migration and invasion, which are crucial for example for tumor metastasis and growth.

2.4.6. Elimination of collagen receptors leads to mild phenotypes *in vivo*

Knock-out mouse models have been constructed to elucidate the α2β1 integrin function *in vivo*. In two independent studies, the α2 integrin subunit deficient mice have been reported to be viable and fertile with only a mild phenotype (Chen *et al.* 2002; Holtkötter *et al.* 2002). A careful analysis of mammary gland branching morphogenesis demonstrated that the complexity of branching was diminished in the α2 integrin null mice (Chen *et al.* 2002). Furthermore, α2β1 integrin was reported to have rather a supportive than essential role in platelet adhesion to collagen (Holtkötter *et al.* 2002). However, other studies indicate that the α2 integrin null mice failed to adhere to type I collagen under either static or shear-stress conditions (Chen *et al.* 2002). To support this, delayed thrombus formation following artery injury have been reported in the α2 integrin null mice (He *et al.* 2003).

Also increased neoangiogenesis has been observed in α2 integrin deficient mice (Grenache *et al.* 2007). The result suggests that α2β1 integrin can repress neoangiogenesis. On contrary, α2β1 integrin has been also found to enhance tumor angiogenesis in a manner dependent on the tumor-specific secretion of angiogenic growth factors (Zhang *et al.* 2008). Additionally, function blocking antibodies against α2 integrin subunit has been shown to repress the pathological angiogenesis (Bix *et al.* 2006). Recent results may explain the discrepancy between the experiments involving α2 integrin deficient mice and α2 integrin inhibitors by demostrating that the α2 integrin deficiency would lead to increased cell surface expression of growth factor receptors that regulate angiogenesis (Zhang *et al.* 2008). Additionally, α2 integrin inhibitors might also affect growth factor receptor signaling (Reynolds *et al.* 2009).

Like α2 integrin deficient mice, mice with an eliminated integrin α1, α10 or α11 subunit are viable and able to reproduce. At first, the α1 gene knock-out mice showed merely a mild decrease in weight (Gardner *et al.* 1996). Later, they were found to have enhanced collagen I synthesis (Gardner *et al.* 1999), hypocellular dermis, deficiency in dermal fibroblast proliferation as embryos (Pozzi *et al.* 1998), reduced tumor angiogenesis (Pozzi *et al.* 2000), increased glomerulosclerosis (Chen *et al.* 2004; Cosgrove *et al.* 2008), defects in cartilage formation, and diminished callus size in a bone fracture model (Ekholm *et al.* 2002). Additionally accelerated aging-dependent development of osteoarthritis in knee-joints (Zemmyo *et al.* 2003), and attenuated atherosclerosis in an α1 / Apo E double knock-out mice (Schapira *et al.* 2005) have

been described. α10 deficient mice had a mild cartilage defect, slightly shortened long bones and mild abnormalities in the growth plate (Bengtsson *et al.* 2005). The elimination of the α11 integrin subunit in mice has been shown to lead to dwarfism and increased mortality due to severely defective incisors (Popova *et al.* 2007). α11 deficiency has been also connected to a reduction in cell proliferation linking the α11 subunit to enhanced tumorigenicity in human non-small-cell lung cancer cells (Zhu *et al.* 2007).

The subtle phenotype of the α2-null mice, and other collagen-receptor integrins, raises the question of collagen-receptor integrin redundancy and compensation *in vivo*. The function of α2β1 integrin may be carried out by other integrins or other cell surface proteins with similar properties. For example, discoidin domain receptors (DDR1 and 2) are receptor tyrosine kinases that act as collagen receptors. Both DDR1 and α2β1 integrins are expressed on epithelial cells (Vogel 1999), however, the elimination of the DDR1 gene has been reported to lead to a more severe phenotype than the lack of α2β1 integrin. For example the majority of the DDR1-null females are infertile (Vogel *et al.* 2001). The fact that αI domain containing integrins have evolved at late stage in the evolution may explain the mild phenotype of the collagen receptor null mice: αI domain integrins may have more important roles in more specific than in fundamental cellular processes.

2.4.7. α2β1 integrin in health and disease

Though the effect of the α2 subunit deletion was reported to be mild, α2β1 integrin function is implicated in a variety of physiological and pathological processes such as immunity, inflammation, autoimmunity, haemostasis, thrombosis and cancer (Zutter and Santoro 2003). As the name VLA-2 designates, α2β1 integrins were initially identified as antigens expressed at the very late stages of T cell activation (Hemler *et al.* 1985). While naïve T cells do not express α2β1 integrin, it is expressed on the subset of activated T cells (Miyake *et al.* 1994; de Fougerolles *et al.* 2000; Rao *et al.* 2000). Inhibiting α2β1 integrin function on T cells has been shown to prevent inflammatory responses in inflammation disorders such as delayed and contact type hypersensitivity and arthritis (de Fougerolles *et al.* 2000). Chronic or long-term activated T cells, or other inflammatory cells expressing α2β1 integrin, have also been connected to complex immune diseases, such as multiple sclerosis, human inflammatory bowel disease and psoriasis (Hafler *et al.* 1985; Lundberg *et al.* 2006; Tsunoda *et al.* 2007; Teige *et al.* 2010). In addition to cells that participate in the adaptive immune system, α2β1 integrin is expressed on the surface of a variety of cells of the innate immune system, such as mast cells (Edelson *et al.* 2004) and natural killer cells (Arase *et al.* 2001). Additionally, α2β1 integrin has been reported to be a cellular receptor for C1q, the first component in the complement cascade and mediator of innate immunity (Edelson *et al.* 2004; Edelson *et al.* 2006). This complex has been detected to act for example in *Listeria monocytogenes* infection (Edelson *et al.* 2006; McCall-Culbreath *et al.* 2008).

Cancer is a complex disease involving several changes in cell physiology. Some cancer types, such as highly invasive melanoma, express elevated levels of α2β1

integrin (Klein *et al.* 1991a; Klein *et al.* 1991b). On the contrary, in mammary carcinoma, as well as in other adenocarcinomas, such as in colon, prostate, skin, lung and pancreatic carcinomas, reduced α2β1 integrin levels correlate with the malignant phenotype (Pignatelli *et al.* 1990; Koukoulis *et al.* 1991; Pignatelli *et al.* 1991; Pignatelli *et al.* 1992; Stallmach *et al.* 1992; Zutter *et al.* 1995; Sun *et al.* 1998; Mirtti *et al.* 2006). *In vivo* models support the idea that α2β1 integrin might have a role in neoangiogenesis (Grenache *et al.* 2007; Zhang *et al.* 2008), additionally, α2β1 integrin is also linked to cell growth and the protection of cells from apoptosis. α2β1 integrin induced gene regulation is also known to affect collagen and collagenase synthesis, features that are required for cell migration and matrix remodeling. Most of these events are characteristic alterations in cell physiology that collectively dictate malignant growth (Hanahan and Weinberg 2000).

In physiological haemostasis following vascular injury, platelets aggregate and form a thrombus in a multi-step process. In atherosclerotic plaques, an excess of collagen produced by smooth muscle cells and fibroblasts, can lead to arterial narrowing (Rekhter 1999). Several studies have suggested that α2β1 integrin is a critical mediator of the platelet interaction with collagen on a damaged vessel wall. This interaction between circulating platelets and collagen may induce the formation of a pathological thrombus. A silent polymorphism on α2β1 integrin is connected with the variant integrin expression levels on the cell surface (Kunicki *et al.* 1997). Epidemiological studies suggest that high α2β1 integrin expression levels on platelets increase the risk of thrombosis (Santoso *et al.* 1999). Additionally, reduced thrombus formation following arterial damage has been reported when α2β1 integrin mediated platelet binding to collagen has been selectively inhibited by small-molecule inhibitors of α2β1 integrin (Miller *et al.* 2009; Nissinen *et al.* 2010). Thus, the α2β1 integrin could provide an attractive therapeutic target to interfere with thrombus formation and other pathological conditions linked to its function.

3. AIMS

Integrins are heterodimeric receptors, which mediate bidirectional signals across the cell membrane. The interaction of α2β1 integrin with the collagenous matrix has been implicated in a number of biological and pathological processes such as thrombosis, inflammation, angiogenesis and wound healing. Thus, understanding the mechanism of the α2β1 integrin inside-out and outside-in activation thoroughly will provide insight into a cellular event of broad biological significance.

The mechanism by which integrin-type collagen receptors recognize the monomeric form of fibril-forming collagens was already well established at the time of the beginning of this thesis project. However, it was not known whether cells interact in a similar manner with the fibrillar form of collagen - the major form of collagen found *in vivo*. The nature of the integrin-type collagen receptors in mediating cell binding to tissue-type collagen fibrils is essential for understanding integrin function *in vivo*.

In addition to its role in mediating cell adhesion to the ECM, and its pivotal roles in cell signaling, α2β1 integrin is also known as a cell surface receptor for some microbial pathogens including human EV1. EV1 binding to α2β1 leads to integrin clustering and consequently the internalization of the virus together with its receptor. In humans, EV1 infections are associated with meningitis, encephalitis, rash, respiratory infections, diarrhea, and even fatal illness in infants. Understanding the mechanism of EV1 infection will be of great importance not only to the field of medical virology, but it might also provide deeper understanding on the mechanism of α2β1 integrin activation.

The specific aims of this doctoral thesis were:

I. To characterize the requirements of α2β1 integrin inside-out activation by studing both receptor clustering and conformational regulation.

II. To study the ability of α2β1 integrin to interact with the fibrillar form of type I collagen and determine its role in collagen fibrillogenesis.

III. To define the structural requirements on α2β1 integrin outside-in signaling.

IV. To uncover the structural basis of α2β1 integrin mediated EV1 entry.

4. MATERIALS AND METHODS

Detailed descriptions of the materials and methods are presented in the original publications I-III.

4.1. INTEGRIN LIGANDS (I, II, III)

Type I collagen fibril formation (II) **–** Type I collagen from bovine skin (Vitrogen®Cohesion or Cellon S.A.) was used at the concentrations of 0.05, 0.1 or 0.5 mg/ml. To initiate collagen fibrillogenesis, samples were subjected to physiological pH and temperature. The progression of fibril formation was followed turbidimetrically with a Beckman DU640 spectrophotometer at 313 nm. For the transmission EM, the fibrillogenesis was allowed to proceed from 60 minutes to 48 hours at 37ºC. Grids embedded with 1.5 % methyl cellulose and stained with 0.4 % uranyl acetate on ice for 10 minutes were finally viewed with a transmission electron microscope (JEM-1200EX, JEOL, Peabody, MA, USA).

EV1 purification (III) **–** EV1 (Farouk strain, American type culture collection; ATCC) was propagated in green monkey kidney cells and purified on sucrose gradients as previously described (Abraham and Colonno 1984). Briefly, the infected cells and the supernatant were collected. Following three freeze-thaw cycles, the virus was precipitated with polyethylene glycol/sodium chloride and purified by ultracentrifugation on 5 - 20 % sucrose gradients.

Ligand matrixes used in the experiments (I, II, III) **–** To study the binding of integrin to collagen fibrils (II), universal binding 96-well microtiter plates (Costar) were coated with the monomeric or fibrillar form of type I collagen (0.5 mg/ml) and stabilized by exposing plates to ultraviolet light for 3.5 minutes. The fibrillogenesis of type I collagen was allowed to proceed for one hour at +37ºC before coating, while the monomeric state was maintained by keeping the temperature of the sample below +4ºC. When a low collagen concentration (0.05 mg/ml) was used, collagen fibril formation was allowed to proceed for 48 hours directly on the wells of a 96-well plate (Nunc) at +37ºC in a humid chamber. When collagen fibrils were not used (I, III), the plates were coated with collagen I, human plasma fibronectin (Chemicon International) or EV1 (5 - 20 $\mu g/cm^2$) o/n at +4ºC.

4.2. INTEGRIN STUDIES AT THE αI DOMAIN LEVEL (II, III)

Human recombinant α1I and α2I domains as glutathione-S-transferase (GST) fusion proteins **–** cDNAs encoding α1I and α2I domains were generated by polymerase chain reaction as described earlier (Ivaska *et al.* 1999a; Nykvist *et al.* 2000) using wild type (WT) human integrin α1 and α2 cDNAs as templates. GST fusion proteins of the integrin α1I domain (amino acids 123-338) in pGEX-4T-3 (Pharmacia) and α2I domain (amino acids 124-339) in pGEX-2T (Pharmacia) were

generated in *Escherichia coli* BL21 cells. The E318W point mutation was introduced into the α2I domain as described (Tulla *et al.* 2008).

Solid phase binding assay for evaluating αI domain affinity to its ligands (II, III) – 96-well microplates (Costar) were coated as described above. Then ligand coated wells were blocked with bovine serum albumin (BSA) containing Delfia® Diluent II (PerkinElmer) for 1 hour at +37ºC. αI domains as GST fusion proteins were allowed to bind their ligands for 1 hour at +37ºC in a concentration series of 10 - 500 nM when binding to collagen was studied, or 0.1 - 20 nM in EV1 studies. αI domain binding was studied in Delfia® Assay Buffer (PerkinElmer) containing 2 mM $MgCl_2$. In the competition studies, 1.5 nM of the α2I-WT domain was allowed to attach to immobilized EV1 in the presence of 0.04 - 0.7 nM of EV1 or 0.01 - 1000 μM of glycine-phenylalanine-4-hydroxyproline-glycine-glutamate-arginine (GFOGER) collagen peptide (Auspep, Australia) synthesized as previously described (Knight *et al.* 2000). Bound αI domains were detected with an Europium^{3+}-labeled (Eu^{3+}) Delfia® GST antibody (PerkinElmer) in the same buffer. Finally, the highly-fluorescent Eu^{3+} label was dissociated with Delfia® enhancement solution (PerkinElmer). The Eu^{3+} signal was determined by time-resolved fluorometry (Victor2 multilabel counter, PerkinElmer). Each assay was performed at least in triplicate and the approximated dissociation constants were obtained by fitting the data into the Michaelis-Menten equation.

Measuring α2I domain-EV1 interaction in real time (III) – Measurements were performed using surface plasmon resonance on a BIAcore-X instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). EV1 was covalently coupled via primary amine groups to the dextran matrix of a CM5 sensor chip (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using sodium formate pH 3.0 as previously described. (Lea *et al.* 1998). Bound EV1 levels were adjusted to about 2000 resonance units. The 1 μM α2I-WT and α2I-E318W GST fusion proteins were run over the chip at a flow rate of 30 μl/min at +25ºC. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HEPES; 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM ethylenediamine tetraacetate (EDTA), 0.005 % surfactant P20; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used as a running buffer throughout the experiment.

Immuno EM studies imaging α2I-WT domain binding to collagen I fibrils (II) – Type I collagen fibrils were spotted onto Formvar/carbon-coated copper EM grids, samples were blocked with 5 % milk in phosphate buffered saline (PBS; 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4; pH 7.4) and the α2I-WT (10 – 300 nM) was allowed to bind to collagen I fibrils for 1 h at +37ºC in the presence of 2 mM $MgCl_2$. Specimens were then fixed and bound α2I-WT was detected by using an antibody against GST-tag in the fusion protein (Amersham Biosciences) and protein-A–gold conjugate (~10 nm; a kind gift from Dr. Posthuma, University Medical Center Utrecht). Finally grids were embedded with 1.5 % methyl cellulose and stained with 0.4 % uranyl acetate on ice for 10 min. Specimens were examined by transmission EM (JEM-1200EX; JEOL, Peabody, MA), and average numbers of bound α2I-WT fusion proteins per collagen fibril D-period were counted. The dissociation constant for α2I-

WT domain binding to fibrillar collagen was estimated by fitting the data to the Michaelis-Menten equation. GST was used as the background control.

Integrin α1I-WT and α2I-WT domains in collagen fibrillogenesis (II) – Collagen I fibrillogenesis (330 μM or 0.1 mg/ml; Vitrogen) was analyzed in the presence of soluble integrin α1I-WT (70 μM) or α2I-WT (15 μM - 330 μM). The fibrillogenesis was followed using a Beckman DU 640 spectrophotometer at 313 nm. The reaction was supplemented either with 2 mM $MgCl_2$ or 2 mM EDTA.

4.3. INTEGRIN STUDIES AT THE CELLULAR LEVEL (I, II, III)

Cell Culture and transfections (I, II, III) – Wild type chinese hamster ovary cells (CHO; ATCC) and cells stably expressing human integrin α1 (Briesewitz *et al.* 1993), α2 (Riikonen *et al.* 1995; Nykvist *et al.* 2000) or the α10 subunit (Käpylä *et al.* 2004) were used (Dickeson *et al.* 1999). Integrin α2 mutations were introduced into the α2 cDNA in the pAWneo2 vector. The mutation α2/α1, where the cytoplasmic domain of α2 integrin has been switched with the corresponding domain of α1 integrin, and the aspartic acid-219/292-asparagine (D219N/D292N) double mutation have been described earlier (Ivaska *et al.* 1999b; Käpylä *et al.* 2000). Tyrosine-410-alanine (T410A), glutamic acid-309-alanine (E309A), glutamic acid-336-alanine (E336A) in the paWneo2 vector and glutamic acid-318-tryptophan (E318W) in a pcDNA™3.1 vector (Invitrogen) were constructed by a modified QuikChange method (Stratagene). Stable cell lines were created using FuGENE6 transfection reagent (Roche Molecular Biochemicals). α2 positive cells were first selected with G418 (0.5 mg/ml; Roche). Additionally, positive cells were stained with integrin α2 mAb 12F1 (BD Biosciences) and flurescein isothiocyanate labeled anti-mouse IgG (DAKO) and isolated by flow cytometry (FACSCalibur, Becton Dickinson). Transfected CHO cells were maintained in α-minimum essential medium (αMEM; Gibco-BRL) supplemented with 10 % fetal calf serum (FCS; PromoCell), 100 IU/ml penicillin-G (Lonza), 100 μg/ml of streptomycin (Lonza) and 500 μg/ml of the neomycin analogue geneticin (G418).

Human osteosarcoma cells, Saos (ATCC), were transfected with the α2-WT subunit (Ivaska *et al.* 1999a) or the α2E336A construct as described above. Transfected Saos cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10 % FCS (PromoCell), 100 IU/ml penicillin-G (Lonza), 100 μg/ml of streptomycin (Lonza) and 250 μg/ml of the G418.

GD25 cells were derived from the embryonic stem cell clone G201, which lacks the integrin subunit β1 (Fässler and Meyer 1995). The cell lines GD25-α2β1 and GD25-α2β1mut (also called GD25-α2β1A and GD25-α2β1Amut; kind gifts from Professor Kristofer Rubin from Uppsala University, Sweden and Professor Deane F. Mosher, University of Wisconsin, USA) stably expressing the WT human integrin α2 subunit and either the mouse β1-WT or a β1 carrying the tyrosine-783/795-phenylalanine (Y783F/Y795F) mutations in its cytoplasmic tail have been described earlier (Wennerberg *et al.* 1996; Wennerberg *et al.* 2000; Grundström *et al.* 2003). Transfected GD25 cells were maintained in DMEM (Gibco-BRL) supplemented with

10 % FCS (PromoCell), 100 IU/ml penicillin-G (Lonza), 100 µg/ml of streptomycin (Lonza), 20 µg/ml puromycin and 50 µg/ml hygromycin.

Human gingival fibroblasts (HGF) were cultured in DMEM (Gibco-BRL) supplemented with 10 % FCS (PromoCell), 100 IU/ml penicillin-G (Lonza) and 100 µg/ml of streptomycin (Lonza).

A short description of cells used in the experiments is given in table 1.

Cell harvesting (I, II, III) **–** Semi-confluent cell cultures were used in the experiments. Cells were detached with 0.01% trypsin and 0.02% EDTA for 3 minutes at room temperature. Trypsin activity was inhibited either by washing the cells with 0.2% soybean trypsin inhibitor (Sigma) in serum-free media or alternatively with serum-supplemented media following repeated washing steps with serum-free media.

Table I *Transfected cell lines*

Cell line	*Construct*	*cDNA sequence (accession no)*	*Reference*
Saos	α2 -pAWneo2 (*1)	nucleotides 1-4559 (X17033.1)	Ivaska *et al*. 1999a
	α2E336A -pAWneo2 (*1)	a point mutation in α2I (X17033.1)	II
CHO	α1 -pLEN (*2)	α1 sequence (X68742)	Nykvist *et al*. 2000
	α2 -pAWneo2 (*2)	nucleotides 1-4559 (X17033.1)	Nykvist *et al*. 2000
	α2E336A -pAWneo2 (*2)	a point mutation in α2I (X17033.1)	II
	α2E309A -pAWneo2 (*2)	a point mutation in α2I (X17033.1)	II
	α2Y410A -pAWneo2 (*2)	a point mutation in α2I (X17033.1)	II
	α2E318W -pCDNA3.1 (*2)	a point mutation in α2I (X17033.1)	III
	α2D219/292N -pAWneo2 (*2)	double mutation in α2I (X17033.1)	Käpylä *et al*. 2000
	α2/α1 -pAWneo2 (*2)	α2 cytosolic sequence switched to α1 (nucleotides 3506-3543)	Ivaska *et al*. 1999b
	α10 -pcDNA3 (*2)	nucleotides 19-3525 (AF074015)	Käpylä *et al*. 2004
GD25	α2 -pPKG-hyg (*3)	human α2	Grundström *et al*. 2003
	β1 -pBS (*4)	murine β1A	Wennerberg *et al*. 1996
	β1Y783/795F -pGEM7Zf (*4)	a double mutation in cytosolic domain of β1A	Sakai *et al*. 1998

(1) cells grown with G418 250 mg/ml

(2) cells grown with G418 500 mg/ml

(3) Brings a hygromycin resistance to GD25-α2β1 cells (0.05 mg/ml)

(4) Brings a puromycin resistance to GD25-α2β1 cells (0.02 mg/ml)

Cellular adhesion assay based on flow cytometry (I) – Collagen I was immobilized on polystyrene microspheres (Ø 9.6 μm) as described (Connors and Heino, 2005). BSA coated beads were used as a negative control. When stated adherent cells were first treated with 10 μM bisindolylmaleimide (Calbiochem) or an α2 integrin inhibitory antibody P1H5 (Santa Cruz) for 15 min at +37ºC. Then, a bead suspension (8 x 10^5 beads) ± 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Calbiochem) was added. Finally, cells were detached and fixed at different time points. Cell adhesion to beads was determined by comparing adherent and nonadherent cell populations by flow cytometry (FACScan, Becton Dickinson; (Connors and Heino 2005).

Cellular adhesion assay based on the determination of viable cells (III) – Nonspecific binding sites were blocked with 1% BSA in PBS for 1 h at +37ºC before addition of 2x10^5 cells / well. When indicated, cells were pretreated with 100 nM TPA (Calbiochem) in serum-free media for 10 minutes. Ethanol was added to the control cells. Cells were allowed to attach for 15 min at +37ºC. Wells were then washed and adherent cells were detected using tetrazolium salt WST-1 reagent (Roche) according to the manufacturer's instructions. Cleavage of WST-1 to the formazan dye by viable cells was detected by measuring the absorbance at 450 nm (Labsystems Multiscan Plus).

Cell spreading assay (I, II, III) – 26,000 CHO cells/cm^2, 30,000 Saos cells/cm^2 or 15,000 HGF cells/cm^2 were added to the collagen or fibronectin coated wells of the microtiter plate. Residual protein sites on all wells were blocked with 0.1% BSA for 1 h at +37°C before the addition of the cells. After 2 hours of cell spreading at +37ºC in serum-free media containing 50 μM cycloheximide (Sigma), the media containing non-adhered cells was poured out and cells were fixed with 4% formaldehyde and 5% sucrose for 30 minutes at room temperature. Cells in sixteen representative fields from four replicate wells were analyzed using phase contrast microscopy (Olympus). The total number of cells attached and the percentage of spread cells were calculated. A spread cell was characterized as having a clearly visible ring of cytoplasm around its nucleus. The morphology of the cells was further analyzed and the percentage of cells that formed long cell projections was determined.

Collagen gel contraction assay (II) – To analyze the capacity of α2 integrins to specifically mediate collagen gel contraction, 300,000 cells per ml of HEPES-buffered (20 μM) collagen I (Cellon, S.A.; 2.4 mg/ml) solution in α-MEM was used. Following collagen polymerization for 2 h at +37°C, the edges of the gels were detached from the sides of the wells, and cell culture media was added. After 72 hours, the surface areas of the gels were measured.

Integrin signaling induced by clustering (I, III) – Cells incubated with 0.1% FCS in DMEM o/n, were treated with an integrin α2 antibody (16B4, Serotec) for 15 min at +37°C, followed by a 15 min – 120 min treatment with the secondary antibody (anti-mouse IgG, DAKO) at +37 °C. Alternatively, cells were incubated with EV1 for 15 min at +37°C. Finally, the cells were collected and analyzed by immunoblotting as described below.

p38 MAPK activation on collagen I and fibronectin coated plates (III) – Cell culture plates were coated with col I (PureCol™, INAMED Biomaterials) or with human plasma fibronectin (Chemicon International Inc.) 20 μg/cm^2 in PBS o/n at +4°C. Before addition of cells, the coated wells were washed with PBS and blocked with 0.1% BSA in PBS for 1 h at +37°C. Cells were allowed to attach in serum-free αMEM at +37°C, 5% CO_2 o/n. Finally, the cells were harvested and the samples were immunoblotted as described below.

Analysis of kinase activation (I, III) – Cells were lysed in Laemmli sodium dodecyl sulphate polyacrylamide gel electrophoresis sample buffer and the sonicated samples were separated on a 10% acrylamide gel and electroblotted onto a Hybond ECL membrane (Amersham Corp., UK). Membranes were probed for activated p38 MAPK as previously described (Ivaska *et al.* 1999b) with a phospho-specific p38 MAPK antibody (threonine-180/tyrosine-182; Cell Signaling Technology or Zymed) and antibodies against total p38 MAPK (Cell Signalling Technology or Zymed) or β-actin (I-19, Santa Cruz). To measure protein kinase Cα (PKCα) activation, a phosphospecific antibody (Millipore) was used. The intensity of the bands was quantified by densitometry using a Microcomputer Imaging Device version M5plus (Imaging Research). Alternatively, p38 MAPK activation was analyzed with flow cytometry using an Alexa Fluor® 488 conjugated P-p38 MAPK (threonine180/tyrosine182) mouse mAb according to the manufacturer's instructions (Cell Signalling). The Cyflogic 1.1.1. (CyFlo Ltd., Turku, Finland) software was used for the analysis of the flow cytometry data.

Analysing α2β1 integrin activation based on an activation specific antibody (I) – 100 nM TPA was added to subconfluent cells in serum-free DMEM. Detached cells were fixed with 4 % formalin and suspended in 3 % BSA in PBS at different time points. Cells were then stained with the mAb recognizing active α2 integrin (12F1, Serotec) or total α2 integrin (16B4, Serotec) and anti-mouse Alexa Fluor® 488 (Molecular Probes). The amount of TPA activated α2 integrin versus total α2 integrin was analyzed by flow cytometry.

Analysis of cell morphology by scanning EM (II) – Cells were allowed to spread for 120 min at +37°C on glass coverslips coated with either monomeric or fibrillar collagen as described above. Cells were fixed with 2.5 % glutaraldehyde for 30 min at +4°C, and specimens were postfixed with 1 % OsO4 for 20 min at +4°C. Finally, samples were dehydrated through ascending concentrations of ethanol, critical point-dried (Tousimis Research Corp.), shadowed by gold sputtering (Hummer VI, Technics West Inc.), and viewed under a Stereoscan 260 scanning electron microscope (Leica Cambridge Ltd, Cambridge, UK) at varying kilovoltage values.

α2β1 clustering and internalization imaged with live cell microscopy (I, III) – Cells were cultured on chambered cover glasses with CO_2 independent medium (Sigma) and analyzed with four-dimensional live cell confocal microscopy (Carl Zeiss Axiovert 100M with LSM510) at 3-min intervals for a total time of 24 min. First, cells were incubated with Alexa-555-conjugated mAb against α2 integrin (16B4, Serotec; labeling kit from Molecular Probes) for 15 min at +37°C. A secondary antibody (goat anti-mouse IgG, Molecular Probes) or 1 μM TPA was added to induce integrin

clustering. For the negative control no secondary antibody was added. Optical slices and volume renderings were visually inspected for the average time of appearance and internalization of at least three clear integrin clusters.

Imaging EV1, α2 and β1 integrin on fixed cells (I, II, III) – α2 integrin clustering was induced either with secondary antibody following an α2 antibody (16B4) treatment or EV1. When PKC -induced clustering was analyzed, cells were treated either with 1 μM TPA or 10 μM bisindolylmaleimide for 40 min at +37°C, or cells were pretreated with bisindolylmaleimide for 15 min followed by the addition of TPA for 30 min. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2 % Triton X-100. To detect EV1, rabbit anti-EV1 (Marjomäki *et al.* 2002) and Alexa-555-conjugated secondary antibodies (Molecular Probes) were used. α2 integrin was stained with mAb 16B4 (Serotec) or 2AE11 (from Dr. Berditchevski, University of Birmingham, Birmingham, UK) and Alexa-488-conjugated secondary antibody (Molecular Probes). Alternatively, α2 integrins were stained during clustering. Three-dimensional ray cast opacity volume renderings and co-localization analyses (automatic thresholding after background subtraction, Costes P-value calculation with 100 iterations) of selected image stacks were performed using the BIOIMAGEXD software (Kankaanpää *et al.* 2006).

Integrin β1 subunits were localized on fixed and permeabilized cells spread on type I collagen fibrils for 120 min. A polyclonal antibody against the cytoplasmic part of the subunit (4080; a gift from Dr Yamada, National Institute of Health) was used. The primary antibody was detected using an Alexa Fluor- 594. Samples mounted with 50 % glycerol were examined with a Zeiss Axioskope 20 fluorescence microscope.

EV1 infection (III) – For the measurement of EV1 infection, Saos-WT, Saos-α2 and Saos-α2E336A cells were incubated with EV1 for 6 h at +37°C and fixed with 3% paraformaldehyde for 20 min at room temperature. 0.2% Triton X-100 permeabilized cells were stained for EV1 and α2 integrin as described above. Finally, cells were examined under a confocal microscope (Carl Zeiss Axiovert 100M with LSM510), and the percentage of EV1 - positive cells was determined by manual counting.

Structural Modeling (I, III) – Structural models for the human α2β1 integrin headpiece domains were based on the crystal structures of the αVβ3 integrin (Xiong *et al.* 2001; PDB ID: 1JV2) and the α2 integrin inserted domain (Emsley *et al.* 2000; PDB ID: 1DZI).

The model for the EV1–α2I domain complex, based on cryo-EM data and the crystal structures of EV1 (Filman *et al.* 1998; 1EV1) and α2I in the closed conformation (PDB ID: 1AOX), was constructed earlier (Xing *et al.* 2004). The EV1 complex with the open α2 conformation was built by superimposing the open form αI domain structure (Emsley *et al.* 2000; PDB ID: 1DZI).

To model clusters of α2β1 bound to EV1, a comparative model of the entire α2β1 integrin heterodimer in the bent conformation was built using the crystal structures of both αVβ3 (Xiong *et al.* 2001; PDB ID: 1JV2) and αIIbβ3 (Zhu *et al.* 2008; PDB ID: 3FCS) as templates. The crystal structure of α2I in the closed conformation (PDB ID: 1AOX) was added. The orientation of the αI domain was modelled manually, constrained by the distance of α2E336 to the βI domain MIDAS site. The extended

conformation of the dimer was modelled by moving the domains by hand to match published electron micrographs.

Structural coordinates were obtained from the RCSB Protein Data Bank (Berman *et al.* 2000) and sequence data from the UniProt online database (29). Sequences were aligned in Bodil v0.8 (Lehtonen *et al.* 2004) and structures modeled using Modeller v7.7 or v9.6 (Martí-Renom *et al.* 2000). Molecular graphics were created using PyMOL v0.98 or v1.1 (DeLano 2002).

Statistics (II, III) – Statistical differences were calculated using two-way analysis of variance. Statistical significance was assigned to $p<0.001$ or $p<0.05$.

5. RESULTS

5.1. INSIDE-OUT SIGNALING PREPARES INTEGRINS FOR LIGAND BINDING (I)

5.1.1. Inside-out activation increases α2β1-mediated cell adhesion to collagen I (I)

Integrin activation is a prerequisite for ligand binding and subsequent cellular signaling. The stimulus for integrin inside-out activation originates from a variety of other cellular receptors. The cellular signaling leads to protein binding to the cytosolic domains of integrin, which initiates the integrin activation. The mechanism of integrin inside-out activation has been a matter of controversy (Bazzoni and Hemler 1998; Carman and Springer 2003; Kim *et al.* 2004). Some reports stress the role of integrin clustering, while other studies emphasize the conformational change in the process. In publication I, the mechanism of α2β1 integrin inside-out activation was studied by mimicking cellular activating signals with TPA. TPA is PKC activator, which mediates the inside-out activation by an unknown mechanism. It has been shown to bind directly to the cytoplasmic tail of the integrin β1 subunit (Ng *et al.* 1999). It has also been suggested to phosphorylate important integrin activators, such as filamin (Tigges *et al.* 2003) or talin which forms a proposed common final step in the integrin inside-out activation process (Litchfield and Ball 1986; Hyatt *et al.* 1994; Tadokoro *et al.* 2003; Tanentzapf and Brown 2006).

To analyze the effect of 100 nM TPA on cell adhesion, CHO cells were transfected with the wild type human α2 integrin subunit (CHO-α2WT). Cell adhesion to collagen I coated polystyrene beads (∅ 9.6 nm) was measured with a flow cytometer at time points up to 60 minutes. The TPA-induced increase in CHO-α2WT adhesion was clear already at the 10-minute time point (I: Fig 2A, B). To verify that the TPA enhanced cell adhesion to collagen I occurred via α2β1 integrin, the CHO-α2WT adhesion was inhibited following pre-treatment with an α2 function blocking antibody, P1H5, and CHO cells transfected with the pAWneo2 expression vector (CHO-pAW) were not able to bind collagen I under any conditions (I: Fig 2E, F). Moreover, the CHO-α2WT cells did not attach to BSA coated beads, which were used as a negative control (I: Fig 2E).

In addition to CHO-α2WT cells, CHO cells transfected either with human α10 or a mutant α2 integrin subunit with an α1 integrin cytoplasmic tail (CHO-α10, CHO-α2/α1) were used to study the role of the integrin α subunit in TPA enhanced cell adhesion. TPA appeared to be able to increase cell adhesion to collagen I irrespective of the swap mutation in the α subunit (I: Fig 2A, C, D). The results indicated that the CHO cell adhesion to collagen I was dependent on collagen receptor integrins, and that the TPA-induced increase was mediated by the β subunit rather than the α subunit.

The importance of the β1 integrin subunit for the TPA-induced inside-out signaling was confirmed using mouse fibroblasts derived from β1 integrin -/- animal stem cells. GD25 cells were transfected to express similar levels of α2β1 integrin or α2β1 integrin

with two point mutations, Y783F/Y795F, in their β1 cytoplasmic tail (GD25-α2β1 and GD25-α2β1mut; Grundström *et al.* 2003). The mutations were designed based on the conserved NPxY motifs in the intracellular domain of integrin β1 subunit. Previously, the mutations have been indicated to alter α2β1 integrin outside-in signaling (Grundström *et al.* 2003). In agreement with previous results, the findings suggest that mutations in the two essential tyrosine residues (Y783 and Y795) do not affect cell binding to collagen I coated beads. However, the TPA-related increase in the GD25-α2β1mut cell adhesion was fully inhibited (I: Fig 3). The results clearly state that inside-out activation is regulated by the integrin β1 subunit in a mechanism that requires NPxY motifs in the cytoplasmic tail of the β1 subunit.

5.1.2. Inside-out activation induces clustering of α2β1 integrins (I)

To uncover the details of the mechanism of the TPA related enhancement of α2β1 integrin-mediated cell adhesion to collagen I, the ability of α2β1 integrins to form clusters without the ligand was studied. Integrin cluster formation was followed with a confocal microscope at 3 minute time-intervals for 24 minutes using human osteosarcoma cells transfected to express α2β1 integrin (Saos-α2). Antibodies, which have been traditionally used to induce integrin clustering (16B4), were used as positive controls. Antibody-induced α2β1 integrin clusters appeared at the 3.5 minute time-point, while the first internalized clusters were seen after 7 minutes, on average. Also TPA-induced PKC activation initiated α2β1 integrin macrocluster formation. Integrin clustering began within ten minutes and the internalization four minutes later. The TPA-mediated clustering and internalization could be prevented with bisindolylmaleimide, a chemical inhibitor of PKCs. This suggests that TPA-induced ligand-free α2β1 integrin clustering requires the activation of PKCs (I: Fig 1A-C). The ligand-free receptor clustering thus appeared to contribute to the α2β1 integrin inside-out activation. Next, the implications of the integrin conformational changes to the process were analyzed.

5.1.3. Inside-out signaling regulates conformational changes in α2β1 integrin (I)

To analyze whether TPA induced the conformational activation in α2β1 integrin, an antibody (12F1) that specifically recognizes conformationally open, active α2I domain, was used (Cruz *et al.* 2005). For the flow cytometric analysis, CHO cells were treated with 100 nM TPA for up to 60 minutes, fixed at different time points, and stained with α2 conformation dependent antibodies. In CHO-α2WT cells total (16B4 positive) α2 integrin expression dropped in the beginning due to α2 integrin internalization. Between 10 to 40 minutes, 12F1 epitopes were exposed reflecting the increase in the number of α2β1 integrins with the open α2I domain conformation (I: Fig 4).

Conformational changes in α2β1 integrin are not, however, restricted to the α2I domain. Instead, large-scale structural rearrangements in the entire heterodimer are required for integrin activation (Alonso *et al.* 2002; Xiong *et al.* 2002; Yang *et al.* 2004). The binding of a ligand has been suggested to cause the conformational change

first in the αI domain. The proposed changes include the downward movement of the α7 helix in the α2I domain. In a model, a single amino acid in the α7 helix acts as an intrinsic ligand for the βI domain in the integrin β subunit. Intrinsic ligand binding to βI then leads to further conformational changes in the entire heterodimer. Finally, the separation of the transmembrane and cytosolic domains initiates integrin signaling. Previously, intermolecular cross-talk has been shown between αM or αL and β2 subunits. In αMI the residue E320 (Alonso *et al.* 2002), and in αLI the residue E310 (Yang *et al.* 2004) have been suggested to serve as critical switches mediating conformational responses. Based on the molecular modeling in study I, amino acid E336 in α2I domain was proposed to act as an intrinsic ligand for the β1I domain (I: Fig 5A; III: 3A).

The mutation E336A was first introduced into the full-length human α2 subunit and transfected into both CHO and Saos cells. Similar expression levels of α2WT and α2E336A integrins in both cell lines were then verified by flow cytometry (III; Suppl. S1). To confirm the expected elimination of conformational modulation in α2E336A, an antibody specific for the active conformation (12F1) was used in the flow cytometric analysis. While in CHO-α2WT cells the 12F1 specific epitopes were invariably exposed following TPA treatment, a similar conformational change was not detected in CHO-α2E336A cells (I: Fig 5D). The results indicated that TPA induced the E336A-dependent conformational activation of α2β1 integrin.

Further analysis revealed that the mutation did not fully inhibit CHO-α2E336A cell adhesion to collagen I coated beads, but the signaling required for cell spreading on collagen I was abolished in the mutants (I: Fig 5B, C; III: Fig 3B, C). Interestingly, TPA-induced α2β1 integrin cluster formation appeared not to be dependent on the E336: similar α2β1 integrin clusters could be seen after 15 min of TPA treatment in both cell types, when cells were stained for α2 (I: Fig 6A,B). Furthermore, TPA caused a corresponding increase in cell adhesion in both CHO-α2E336A and CHO-α2WT cells. Thus, integrin inside-out signaling appeared to be synergistically regulated by α2β1 integrin clustering and conformational activation. Activated integrins have the capacity to bind their ligands and induce cell signaling. Next, the structural requirements of α2β1 integrin binding to its ligands, the fibrillar form of collagen I and EV1 were studied.

5.2. α2β1 INTEGRIN IS A RECEPTOR FOR COLLAGEN FIBRILS (II)

Prior to this thesis project, several studies had shown how integrin-type collagen receptors recognize the monomeric form of fibril forming collagens. However, it was not known whether cells interact in a similar manner with the fibrillar collagen - the major form of collagen found *in vivo*. In publication II, the α2β1 integrin-mediated recognition of collagen I fibrils was carefully examined.

5.2.1. Cells form long protrusions upon spreading over collagen fibrils (II)

Type I collagen fibrils were formed for the experiments by raising the temperature and pH of a monomeric collagen solution to physiological level (+37°C; pH 7.4). The

quality of fibrils was analyzed by negative-staining of the samples adsorbed onto Formvar/carbon-coated EM grids. The fibrils appeared to have a characteristic D-periodic banding pattern, and to be indistinguishable from those found in tissues. However, fibrils with a loose structure were also seen (II: Fig 1A, B). Next, the integrin-mediated cell spreading and the morphology of spread-out cells were analysed.

Cell spreading not only requires specific attachment of receptors to their ligands, but also sophisticated cell signaling. CHO-α2WT cells appeared to form characteristic broad lamellipodia when spread onto monomeric collagen I. However, on fibrillar type I collagen the cells behaved differently: long extensions were seen when CHO-α2WT cell-spreading was examined by scanning EM (II: Fig 4A-B). In addition to CHO cells, human osteosarcoma cells (Saos WT), endogenously expressing α1β1, α10β1 and α11β1 integrins, Saos-α2β1 cells, and human gingival fibroblasts (HGF) were allowed to spread on a matrix of fibrillar collagen I. Analysis of these cells revealed that the formation of cellular projections was not dependent on α2β1 integrins, though α2β1 could clearly promote the process (II: Fig 5A-C). The β1 integrins were shown to participate in the formation of long cellular extensions, and clear β1 clusters could be seen along projections in HGFs spread for 120 min on fibrillar collagen I. The β1 clusters were visualized by immunostaining using an antibody (4080) against the cytoplasmic domain of β1 integrin (II: Fig 3). The formation of cellular projections could be prevented by a function-blocking antibody against the integrin β1 subunit (data not shown). Thus, based on the analysis of several different cell types, it was evident that fibrillar collagen induced β1 integrin-dependent formation of long cellular projections.

To further characterize the interactions of α1β1 and α2β1 integrins with fibrillar collagen I, CHO-α1WT and CHO-α2WT cells were allowed to spread on type I collagen fibrils and monomers for 120 min. It appeared that CHO-α2WT cells were able to spread on both monomeric (76 % ± 8.7 %) and fibrillar collagen I (64 % ± 6.9 %), while α1β1 integrins could mediate CHO cell spreading only on collagen monomers (26 ± 2.7 %; fibrils 8.4 % ± 1.9 %) (II: Fig 2). When the cells were cultured inside the floating collagen I gel for 72 h, once again CHO-α2WT cells could bind and reorganize the surrounding collagen fibrils into a more dense and compact arrangement than did CHO-α1WT cells. Next, CHO cells harbouring the double mutation D219N/D292N in the α2I domain were used. The mutations have been designed close to the MIDAS motif in a site not essential for Mg^{2+} binding, and the double mutation has been previously shown to prevent α2β1 integrin-mediated cell spreading (Käpylä *et al.* 2000). In publication II, it was shown that the α2D219/292N is also unable to mediate the collagen gel contraction (II: Fig 6A-B). The results supported the idea that tight, α2β1 integrin-mediated contacts between fibrils and integrins are required for the collagen fibril recognition and gel contraction. Most importantly, the α2β1 integrin appears to be a functional receptor not only for type I collagen monomers but also for collagen fibrils.

5.2.2. α2β1 integrin specifically recognizes also collagen fibrils (II)

To compare the affinities of the α1I and α2I domains to monomeric and to fibrillar type I collagen, both forms of collagen I were immobilized on a microtiter plate. Wild type α1I and α2I domains were used as GST-fusion proteins (α1I-WT, α2I-WT). In the assay, αI-GST fusion proteins were allowed to bind their ligands at concentrations between 10 nM and 500 nM. Bound αI-GST fusions were detected with an Eu^{3+} labeled antibody against GST. Finally, the label was dissociated into an enhancer solution, in which it forms highly fluorescent complexes. The fluorescence was measured in a time-resolved fluorometer. As a result, the K_d value calculated from the saturation curve appeared to be in good agreement with previously reported values for α1I-WT and α2I-WT domain binding to the monomeric form of collagen I (α1I-WT: $K_d \approx 30 \pm 3$ nM; α2I-WT: $K_d \approx 10 \pm 1$ nM). However, both of the αI domains studied here bound fibrillar collagen with affinities, which were about ten times weaker than those for monomers (α1I-WT: $K_d \approx 250 \pm 30$ nM; α2I-WT: $K_d \approx 100 \pm 10$ nM). The binding was not only weaker but also the total number of bound αI domains appeared to be reduced following collagen fibril formation (II: Fig 7A, B).

To ensure that α2I-WT domain really bound to the classical D-periodic type I collagen fibrils, the binding was visualized by an indirect immunogold labeling technique using antibodies against the GST-tag of the fusion protein. For the analysis, collagen I fibrils were adsorbed onto EM grids. Electron micrographs showed a clear concentration dependent binding of α2I-WT domain in the characteristic D-periodic banding pattern (67 nm). When the binding data was fitted to a Michaelis-Menten equation, the results appeared to be comparable to the solid phase binding assay, the K_d being $\approx 62 \pm 2$ nM. No immunogold particles were observed in negative controls treated with GST only instead of the α2I-WT domain as a fusion protein (II: Fig 8A-D). ImmunoEM analysis together with the solid phase binding assay indicated that the α2I-WT domain is able to specifically bind to tissue-type collagen fibrils.

5.2.3. Soluble α2I-WT domains might modulate collagen fibrillogenesis (II)

While both α1I-WT and α2I-WT domains appeared to bind to monomeric collagen with higher affinities than to collagen fibrils, it was intriguing to study whether α1I-WT or α2I-WT would engage with collagen I fibril formation. When soluble αI domains were mixed with collagen I monomers under physiological conditions and collagen fibril formation was followed spectrophotometrically at 313 nm, there was a clear difference between the α1I-WT and the α2I-WT domains. The results indicated that 70 μM α1I-WT domain first induced fibril formation but later completely inhibited the process. Instead, the α2I-WT domain prevented fibril formation in a concentration dependent manner: high concentrations (α2I-WT: 330 μM; collagen I: 330 μM) inhibited the process right from the very beginning. Though the conditions did not mimic the *in vivo* situation, where integrins are bound to the cell membrane, the result indicated that the α2I-WT domain binds to a collagen I monomer stronger than collagen I monomers bind to each other.

5.3. COLLAGEN I AND EV1 BIND α2β1 WITH HIGH AFFINITY (II, III)

In addition to collagen I, EV1 recognizes human α2 integrin to initiate its life cycle in the host cell. Previously, EV1 has been shown to bind to the α2I domain with an affinity 10-fold higher than to collagen I (Xing *et al.* 2004). It is well known that the α2I domain exists in two conformations: open and closed. A conformation change from the closed to the open form is thought to increase the affinity of α2I domain to the ECM ligands collagen and laminin (Aquilina *et al.* 2002; Tulla *et al.* 2008). Before this thesis project, it was not known whether virus entry depends on, or induces, changes in the α2I domain activation state. In study III, further insight in the mechanisms of the α2β1 integrin ligand recognition was gained.

5.3.1. Both collagen and EV1 may cluster α2β1 integrins (III)

Ligand-induced integrin clustering is a well known phenomenon (Bazzoni and Hemler 1998; van Kooyk *et al.* 1999; Constantin *et al.* 2000; van Kooyk and Figdor 2000; Hogg *et al.* 2002; Li *et al.* 2003a). In publication III, the EV1-induced α2β1 integrin clustering was imaged by confocal microscopy. Within 15 min, EV1 and the α2-specific antibodies were shown to induce similar α2β1 integrin macroclustering (III: Fig 4A; 6). α2 antibodies were used as positive controls to mimic natural integrin clustering. To gain a deeper understanding of the process, molecular modeling was applied to study whether collagen fibrils would act as multivalent ligands and induce the formation of integrin clusters. The molecular model (Fig 9) has been created by Dr. Santeri Puranen, and part of the model has been earlier published in his thesis project (Puranen 2010).

Several high-affinity integrin binding motifs have been reported to be present in collagen monomers (Knight *et al.* 1998; Raynal *et al.* 2006). Three of these binding sites, GLOGER, GFOGER and GASGER, appear to be recognized with significantly higher affinities than are other regions (Fig 9A; Xu *et al.* 2000). These sites are located at 60-70 nm, 145-155 nm and 250-260 nm from the C-terminus of the monomer. While the length of the integrin extracellular domain is 18 nm and the maximal separation from the integrin leg part is approximately 15 nm at the plasma membrane (Fig 9B), it can be concluded that a single collagen monomer cannot, without bending to an unreasonable degree, bring integrins into contact with each other. Thus, it is unlikely that monomeric collagen alone induces clustering simply by mechanical means. Collagen fibers, which can extend to several hundreds of nanometers in diameter may, however, present integrin-binding motifs with a spacing, whereby the cytoplasmic parts of the bound integrins would make contact with each other, or at least be in close proximity to each other (Fig 9C).

To summarize the results, there is evidence that EV1 clusters α2β1 integrins and, in contrast to the properties of collagen monomers, collagen fibrils appeared to fulfill the geometrical requirements for a multivalent ligand able to activate receptor clustering.

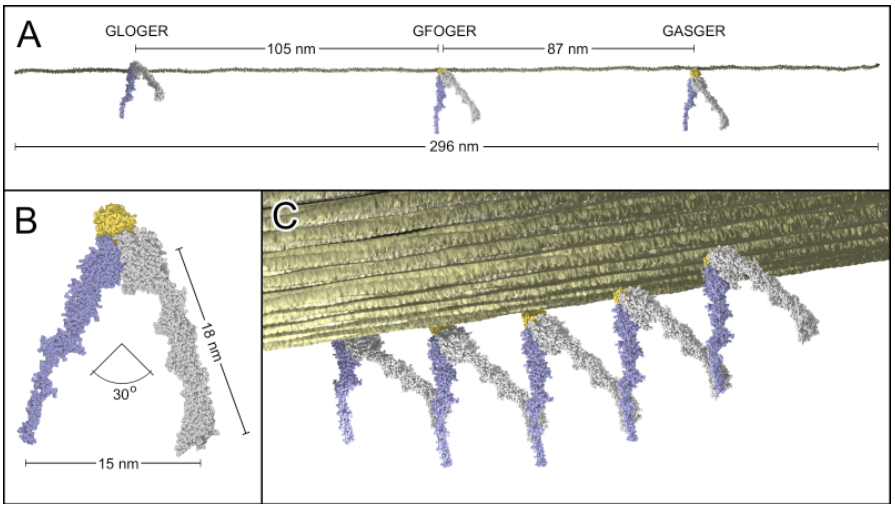

Figure 9. ***Collagen I monomers are unlikely to induce integrin clustering, whereas collagen fibers may do so by bringing receptors into contact with each other.*** The high-affinity α2β1 integrin binding motifs GLOGER, GFOGER and GASGER are separated along the collagen I monomer (A). The extracellular region of active α2β1 integrin is portrayed in all figures. When the integrin is in the active conformation and its leg parts are fully separated, the angle between the leg regions is 30 degrees (B). Figure A shows that collagen I monomer cannot mechanically bring two or more active α2β1 integrins into contact with each other without bending significantly. In a collagen fiber, however, high-affinity binding motifs on neighboring microfibers may be located close to each other due to the monomer stagger and specific cross-linking. Thus, a collagen fiber may induce integrin clustering by bringing integrin receptors into close proximity (C). A 50 nm diameter collagen fiber with several bound α2β1 integrins is portrayed (C). Molecular modeling by Santeri Puranen.

5.4. EV1 AND COLLAGEN I EXPLOIT α2β1 IN A DIFFERENT MANNER (III)

Integrin α2I domain has been thought to exist mostly in a closed, low-affinity conformation. In one of our earlier publications based on cryo-EM and molecular modeling, the closed α2I domain was suggested to form an intimate contact with the outer canyon wall of the EV1 capsid (Xing *et al.* 2004). Amino acid residues on the α2I surface, 199-201, 212-216 and 289, which have been shown by mutagenesis to be essential for EV1 recognition (King *et al.* 1997; Dickeson *et al.* 1999), appeared to form favourable electrostatic contacts with the negatively charged residues on the virus capsid. In this thesis project, the open form of α2I was modelled in a complex with EV1 by superimposing the described model of α2I (closed)-EV1 complex. First, it should be pointed out that the conformational change from the closed to the open form appeared not to alter the EV1 virus binding surface on α2I domain. The largest

conformational changes on the α2I domain surface were seen on the opposite side of the EV1 binding site. However, the residue Asn289 was positioned in the middle of the αC helix, which has been suggested to undergo extensive structural rearrangements. Based on the model, it could be hypothesized that both α2I (closed) and α2I (open) are able to bind the virus. The location of Asn289 might, however, propose that EV1 binding could favour the closed instead of the open form of α2I domain (III: Fig 1A).

As described previously, ligand binding is typically accompanied by extensive conformational changes in α2I domain, creating an open, high-affinity domain. During the conformational change, the α7 helix undergoes a displacement. The residue Glu318 is located in this helix and during the conformational change, it moves from a buried to an exposed position. Mutating the glutamic acid at the position 318 to tryptophan has been shown to create a constitutively active α2I domain, α2I-E318W (Aquilina *et al.* 2002; Tulla *et al.* 2008). The binding of the α2I-GST fusion proteins, α2I-WT and α2I-E318W, to immobilized EV1 was analyzed in a Eu^{3+} based solid phase binding assay as described above. Consistent with the result obtained from the molecular modeling of the α2I–EV complex, EV1 formed a higher affinity complex with the closed α2I-WT domain than with the constitutively active α2I-E318W domain (α2I-WT: $K_d \approx 0.8 \pm 0.2$ nM; α2I-E318W: $K_d \approx 3 \pm 0.2$ nM; III: Fig 1C). As expected the α2I-E318W domain had higher affinity to monomeric type I collagen than did α2I-WT domain (α2I-WT: $K_d \approx 39 \pm 3.5$ nM; α2I-E318W: $K_d \approx 7 \pm 0.5$ nM; III: Fig 1B).

To analyze the interaction of α2I-WT domain with the virus in real time, the surface plasmon resonance based technology from BIAcore was used. First, EV1 was immobilized through the primary amine groups to the dextran matrix of a sensor chip and α2I-WT and α2I-E318W domains were passed over the chip. The replacement of binding buffer with any regenerating buffer did not cause dissociation of the EV1 bound to α2I domain, indicating a strong interaction between the α2I domain and EV1. Thus, sequential injections of increasing concentrations of α2I domain in one continuous analysis cycle were performed. The results failed to fit the 1:1 binding model (Karlsson *et al.* 2006) indicating that EV1 harbors more than one kind of binding sites on α2I domain (data not shown).

When binding of the α2I domain to EV1 at a concentration of 1 μM was further analyzed, both α2I-WT and α2I-E318W domains appeared to associate with immobilized EV1 in a fast and tight fashion. However, when the dissociation phase was analyzed, it became apparent that the dissociation of α2I-E318W domain was faster than the dissociation of α2I-WT domain (III: Fig 1D). The result partially explains why at the 1 hour time point in the solid phase binding assay the α2I-WT domain seemed to bind tighter to EV1 than did α2I-E318W domain. To verify the results, the binding of 1.5 nM of the α2I-WT domain to immobilized EV1 was analyzed in the presence of a concentration series of either soluble EV1 (0.04 -0.7 nM; III: Fig 1E) or GFOGER (0.01-1000 μM; III: Fig 1F) in a Eu^{3+} based solid phase binding assay. The collagenous peptide containing the integrin recognition site, GFOGER, inhibited the adhesion of α2I-E318W domain to EV1, while soluble EV1 inhibited the adhesion of α2I-WT domain. The results clearly indicate that the conformational change to the open form improves the binding of the α2I domain to

collagen while EV1 prefers the closed conformation. Additionally, two independent analyses with increasing concentrations of soluble EV1 (Mw 5.65 x 10^6) binding to an α2I-WT domain as a GST fusion protein (Mw 4.95 x 10^4) indicated that under these conditions 7-15 % of 60 putative integrin binding sites per virion were occupied (III: Fig 1F).

EV1 recognized α2I-WT domain better than a constitutively active α2I-E318W domain not only on the αI domain level but also when the point mutation was introduced into the full length α2 subunit and expressed on the surface of CHO cells (CHO-α2E318W). Cell adhesion to immobilized collagen I was clearly increased due to the mutation ($p<0.001$), while the mutation decreased cell binding to immobilized EV1 ($p<0.05$). Cell binding was detected at the 15 min time point using the WST-1 reagent (III: Fig 2). All the results so far support the tendency of EV1 to favour an inactive α2β1 integrin.

5.4.1. A bent α2β1 integrin mediates cell binding to EV1 but not to collagen I (III)

In publication I, the E336A mutation of α2 integrin was shown to inhibit the intersubunit communication between α2 and β1 subunits (I: Fig 5B, C, D; III: Fig 3B). The corresponding mutation in αL integrin has been reported not only lacking in conformational activation, but to also shift equilibrium from the extended integrin conformation towards the bent conformation (Salas *et al.* 2004). EDTA, a chelator of divalent cations, has been reported to have a similar effect on integrin conformation (Xie *et al.* 2004). In this thesis project, the point mutation E336A was shown to markedly reduce α2-mediated CHO cell adhesion to immobilized collagen I. In contrast, cell binding to immobilized EV1 significantly increased due to the mutation (III: Fig 3C). Similar effects were seen in CHO-α2WT cells when cell binding to immobilized collagen I and EV1 were measured in the presence of 2 mM EDTA: the chelator inhibited cell adhesion to collagen I and increased adhesion to EV1 (III: Suppl Fig S2A). In all situations, TPA-induced α2β1 integrin clustering and conformational change further increased cell adhesion to its ligands (III: Fig 3D). The enhancement in adhesion was, however, more pronounced when collagen I served as a ligand: collagen I binding appeared to benefit from both clustering and conformational change while EV1 receptor recognition only appeared to require receptor clustering.

5.4.2. Collagen activates p38 MAPK in an α2E336 -dependent manner (III)

To initiate the integrin heterodimer specific signal transduction following ligand binding, large allosteric changes in integrin conformation have to take place. To enable signaling, connections between the cytoplasmic tails of integrin α and β subunits, i.e. the bridges holding the transmembrane and cytosolic domains together, need to be broken. p38 MAPK activation by phosphorylation has been linked to α2β1 integrin signaling in several different cell lines (Ivaska *et al.* 1999b; Ravanti *et al.* 1999; Xu *et al.* 2001; Bix *et al.* 2004; Mazharian *et al.* 2005). The mechanism of heterodimer specific signaling of α2β1 integrin was explored in study III.

Integrin clustering has been suggested to be a prerequisite for subsequent signaling. As a result of antibody-mediated clustering, CHO-α2WT and Saos-α2WT cells induced p38 MAPK phosphorylation transiently peaking at 15 minutes. The activation of the kinase was analyzed using p38 MAPK antibodies specific for the phosphorylated kinase on immunoblots (III: Fig 4B, C), and in flow cytometry based applications (III: Fig 4D). Under all experimental conditions, the primary or secondary antibody alone did not induce α2β1 integrin clustering and consequent p38 MAPK phosphorylation.

When CHO-α2WT and CHO-α2E336A cells were plated on collagen I or on fibronectin, α2β1 integrin-mediated p38 MAPK activation appeared to be clearly dependent on conformational changes in the receptor: in CHO-α2WT cells both collagen I and fibronectin induced phosphorylation while in CHO-α2E336A cells p38 MAPK was activated only on fibronectin (III: Fig 5A). Similar results were obtained following 15 minutes of antibody-mediated clustering in both α2β1 integrin expressing CHO (III: Fig 5B) and Saos cells (III: Fig 5C). The results indicate that in addition to α2β1 integrin clustering, p38 MAPK phosphorylation requires the conformational regulation of the α2β1 integrin. This was confirmed when the E336A mutation prevented the α2β1 integrin clustering induced activation of the kinase (III: Fig 5B, C). Additionally, an EDTA-induced bent α2β1 integrin conformation inhibited the p38 MAPK activation in CHO-α2WT cells (III: Suppl Fig S2A). EV1-mediated clustering also appeared not to support p38 MAPK activation (III: Fig 6 A,B). Thus, EV1 neither requires the conformational activation of α2β1 integrin for receptor recognition, nor does induce it during cell entry.

5.4.3. Both collagen I and EV1 activate PKCα in an α2E336 -independent manner (III)

In our previous experiments, EV1 was shown to require PKCα activation to enter the host cell (Upla *et al.* 2004). Similarly, PKCα phosphorylation has been reported to be induced by α2β1 integrin antibody clustering (Upla *et al.* 2004). While EV1 did not induce p38 MAPK activation, which was previously demonstrated to require both a conformational change in α2β1 integrins and clustering of them, it could be proposed that the mechanisms to initiate the α2β1 integrin-mediated PKCα and p38 MAPK signaling pathways differ from each other. Indeed, in contrast to p38 MAPK activation, PKCα phosphorylation appeared not to require the E336 linked conformational regulation of α2β1 integrin. PKCα activation was induced following a 30 min treatment with EV1, and shown by immunoblotting the samples with a PKCα specific antibody (III: Fig 6C). Consistent results were obtained when EV1 infected cells were analyzed by labelling EV1 in paraformaldehyde fixed cells 6 h post infection: the E336A mutation in the α2 integrin did not affect the ability of EV1 to infect host cells (III: Fig 6D). Thus, the conformational regulation was neither required for EV1 signaling nor entry.

5.4.4. EV1 forms a unique ring-like α2β1 cluster (III)

All the observations in publication III indicate that EV1 is able to bind to α2β1 integrin molecules, which have closed α2I domains, and which have adopted a bent conformation. While the ECM ligands were not able to mediate outside-in signaling through the bent α2β1 integrin conformation, this did not prevent EV1 cell entry. To further test the hypothesis that EV1 binds and clusters the bent α2β1 integrin, the structural models of α2β1 heterodimers in the bent and the extended conformations were constructed by molecular modeling (III: Fig 7A-D).

An icosahedral EV1 capsid is formed of 12 pentamers, each of which has binding sites for five α2β1 integrins. Previously it has been demonstrated that a single pentamer on the EV1 capsid is able to accomodate five α2β1 integrins in the extended conformations without steric hindrance (III: Fig 7A; (Xing *et al.* 2004). Similarly, in publication III adjacent integrin binding sites were shown to be also able to bind five bent α2β1 integrins (III: Fig 7B). This model, however, assumed the very close packing of heterodimers and required flexibility in the α2β1 integrin structure. In an alternative model, bent integrins are located around the virus in a way that does not accommodate five binding sites in one pentamer, but uses adjacent pentamers (III: Fig 7C, D). The ideal model, where the integrins are symmetrically arranged, was proposed to require the cell membrane to be curved around the viral particle.

While only 10 out of 60 putative integrin binding sites on the viral capsid were demonstrated to be able to be occupied simultaneously (III: Fig 1E), the stoichiometry supported the alternative model for EV1-induced α2β1 integrin clustering (III: Fig 7C, D). According to the model, the arrangement of integrin cytosolic domains in a cluster was suggested to form a unique ring-like structure. The results presented in publication III suggest that EV1 clusters bent, inactive α2β1 integrins, without triggering the conformational activation of the receptor. Altogether, EV1 seemed to activate its own entry only by clustering α2β1 integrins in a process that required PKC activation.

6. DISCUSSION

6.1. α2β1 INTEGRIN INSIDE-OUT SIGNALING (I)

α2β1 integrins contribute to the regulation of physiological processes such as immunity, inflammation and haemostasis, and thus also to the development of diseases including autoimmunity, thrombus formation and cancer progression (Zutter and Santoro 2003). Consequently, α2β1 integrins are targets in developing drugs for the treatment of various pathological conditions where α2β1 integrins are involved. To design effective drugs, it is crucial to thoroughly understand the molecular mechanism of α2β1 integrin function.

The results presented in publication I suggest that in α2β1 integrin inside-out activation two mechanisms, clustering and conformational modulation, are synergistically regulated. According to the conventional hypothesis, integrins are maintained in a bent low-affinity conformation in resting cells. And in contrast, extended integrins have been suggested to be active, able to bind their ligands and induce cell signaling. Integrins not only strengthen the interaction with their ligand by going through conformational changes, but also by laterally migrating on the cell membrane forming integrin clusters. Before this thesis project most of our knowledge of the integrin structure-function relationship was based on β2 and β3 integrins (for reviewes see Springer and Wang 2004 and Arnaout *et al.* 2005).

The allosteric conformational regulation of integrins has been proposed to be similar for all integrins independent of the presence or absence of the αI domain (Alonso *et al.* 2002; Xiong *et al.* 2002; Yang *et al.* 2004). In α2β1 integrin, the conformational modulation was shown to be critically dependent on residue E336 in the α7 helix of the α2I domain. Based on molecular modeling the amino acid E336 was suggested to act as an intrinsic ligand for the β1 subunit and to induce re-coordination of the metal ion in the β1I domain. This is consistent with the mechanism described for αLβ2 and αMβ2 integrins (Alonso *et al.* 2002; Yang *et al.* 2004). It has been suggested that to enable the critical glutamate (E336 in α2, E310 in αL and E320 in the αM subunit) to interact with the βI domain, the α7 helix in the integrin α subunit moves downward. Correspondingly, mutations that stabilize the α7 helix in the downward position have been reported to create a high-affinity integrin (Mould *et al.* 2003; Hato *et al.* 2006; Cheng *et al.* 2007). And in agreement with these findings, the low-affinity α2β1 integrin conformation caused by the mutation E336A in the α2 subunit markedly reduced α2β1 integrin-mediated cell binding to collagen I and prevented CHO cell spreading on collagen I. It has been suggested that the corresponding mutations in the αL and αM integrin subunits adopt a low-affinity conformation (Alonso *et al.* 2002; Yang *et al.* 2004). In contrast to the effects of the glutamate mutations in α2β1 and αMβ2 integrins, the mutation E310A in αLβ2 integrin has been reported to prevent ligand binding entirely (Alonso *et al.* 2002; Yang *et al.* 2004).

Integrin clustering and conformational modulation, i.e. changes in integrin valency and affinity, are generally linked to the regulation of receptor activation. Distinguishing changes in integrin valency from changes in affinity is essential for understanding the mechanism of α2β1 integrin activation. Furthermore, information of conformational modulation may impact the development of therapeutic compounds designed to modulate integrin activity to treat pathological conditions. The conformational regulation of αLβ2 and αIIbβ3 integrins during inside-out activation has been proposed to be responsible for integrin activation, and clustering only follows ligand binding (Buensuceso *et al.* 2004; Kim *et al.* 2004). In contrast to the lacking αLE310Aβ2 integrin adhesion to its ligand, intercellular adhesion molecule-1 (Kim *et al.* 2004), TPA-induced clustering appeared to be sufficient to achieve α2E336Aβ1 integrin-mediated cell binding to type I collagen even in the absence of conformational regulation. The results published in study I also emphasize the order of events, stating that α2β1 integrin clustering would precede, not follow the global conformational regulation of the receptor. In agreement with the results in publication I, there are also other studies, which highlight the significance of integrin clustering in integrin inside-out activation (van Kooyk and Figdor 2000; Hogg *et al.* 2002; Bunch 2010).

Some reports have stressed the role of conformational changes in integrin affinity during inside-out activation and speculated that integrin clustering would occur only after receptor binding to multivalent ligands (Carman *et al.* 2003; Carman and Springer 2003; Kim *et al.* 2003; Kim *et al.* 2004). In contrast, other reports emphasize it is not the integrin clustering, but conformational changes that take place after ligand binding (Bazzoni and Hemler 1998). Publication I suggested that the conformational changes in α2β1 integrin follow receptor clustering during inside-out activation. Furthermore, the results demonstrate that both of these changes take place subsequent to TPA treatment and do not require the presence of a ligand. Consistent with this, there is also a study, which reports the formation of αLβ2 integrin clusters as a prerequisite for dynamic monocyte extravasation. The report stressed the role of both ligand-independent clustering and conformational modulation in αLβ2 integrin function (Cambi *et al.* 2006). These results are in agreement with the model for α2β1 integrin activation on the surface of adherent cells as presented in publication I. A rapid integrin response is crucial for circulating platelets and lymphocytes (Hato *et al.* 1998; Constantin *et al.* 2000). Although α2β1 integrins on adherent cells may behave otherwise differently, the strength of the integrin binding increases when α2β1 integrins are in close proximity on the cell surface. Consequently, each individual integrin may be able to bind a relatively stiff collagen fibril, which presents multiple integrin binding sites (Xu *et al.* 2000; Kim *et al.* 2005). In synergy, α2β1 integrins would mediate a tight overall cell adhesion to collagen fibrils.

Controlling the activation of α2β1 integrins via an inside-out signaling mechanism was shown to involve both clustering and conformational regulation, but the molecular basis for the signaling events still remains elusive. The cellular environment is very complex; by now at least 41 different cytoskeletal proteins that are associated with the short cytoplasmic tails of integrin β subunits at least some situations have been reported (Legate and Fässler 2009). Integrin adhesion to the extracellular matrix is regulated by cytoskeleton associated proteins such as talin and kindlin (Tadokoro *et al.*

2003; Anthis *et al.* 2009). PKC activation has been suggested to induce the association of talin with the cytosolic domain of the β subunit (Kupfer *et al.* 1990; Han *et al.* 2006). Alternatively, PKC may interact directly with the β subunit (Ng *et al.* 1999). While TPA-mediated PKC activation was shown to increase α2β1 integrin clustering, clustering has also been linked to PKC activation (Upla *et al.* 2004); publication III). Thus, the events seem to be intimately linked: PKC activation promoted α2β1 integrin clustering, which further increased PKC activation. In other words, the same signaling proteins that are activated by ligand-mediated clustering may also initiate ligand-free clustering. The TPA-mediated inside-out activation appeared not to be α subunit specific. Instead, the binding was dependent on a conserved NPxY motif in the β1 subunit. All the results stressed the role of the β1 subunit in the regulation of α2β1 integrin during TPA-induced inside-out activation, and other collagen receptors may also be activated by similar mechanisms.

6.2. α2β1 INTEGRIN AS A RECEPTOR FOR COLLAGEN I (II)

Inside-out signaling leads to integrin activation, which in turn favors ligand binding (Lu *et al.* 2001b; Beglova *et al.* 2002; Takagi *et al.* 2002). A crystal structure of the α2I domain in complex with a triple-helical collagenous peptide has revealed the structural basis of α2β1 integrin ligand binding. Binding has been suggested to be dependent on the critical glutamate within a collagenous GFOGER motif that would act as a cation coordinating residue (Emsley *et al.* 2000). Although details of integrin binding to the collagenous peptide or the monomeric form of collagen are available, publication II provided, for the first time, information about the interaction of α2β1 integrin with a collagen I fibril. Furthermore, the molecular modeling of α2β1 integrin binding to a collagen fibril, as presented in this thesis, proposes that collagen fibrils, unlike monomers, are able to act as multivalent ligands and induce the clustering of collagen receptors. The results also suggested a role for the α2I domain in collagen fibrillogenesis.

The results in publication II propose that α2β1 integrin is a functional receptor also for collagen I fibrils. However, collagen fibril formation may lead to conformational changes in the α2β1 integrin recognition sites on collagen I. Moreover, several integrin binding sites were suggested to be hidden inside the fibrillar collagen structure during fibrillogenesis. The first crystallographic determination of the collagen type I supermolecular structure supports the hypothesis that not all integrin recognition sites are available for integrin binding (Orgel *et al.* 2006). A subsequent study based on the structural model demonstrated that the integrin binding sites GROGER and GLOGER would allow α2β1 integrin binding on fibrillar collagen without a steric barrier. GFOGER and GMOGER may also be accessible, while GQRGER and GASGER are completely buried within the fibril (Herr and Farndale 2009). Consequently, α2β1 integrin preferentially binds to collagen monomers, where all the integrin binding motifs are available. Despite the availability of the α2β1 integrin binding sites on monomeric collagen, the molecular modeling presented in this study suggests that a single collagen monomer cannot bring integrin receptors into contact with each other without bending to an unreasonable degree. Thus, monomeric collagen alone is

unlikely to induce α2β1 integrin clustering by a simple mechanical means. However, neither the crystallographic determination nor the molecular modeling exclude the possibility that α2β1 integrins would also be able to bind to collagen I fibrils and anchor the cells to the surrounding ECM. Furthermore, the integrin binding sites on a collagen fibril are able to induce α2β1 integrin clustering required for integrin signaling. However, in tissues the integrin-mediated cell binding to the ECM is far more complex and integrin binding to collagen fibrils may be enhanced by proteins, such as fibril associated collagens. Collagen receptor integrins have been reported to recognize for example type IX collagen, which is typically associated with type II fibrils in cartilage. Type IX collagen thus provides an indirect mechanism for cells to bind cartilaginous matrix via the collagen receptor integrins (Käpylä *et al.* 2004).

6.2.1. α2β1 integrin may control collagen I fibrillogenesis

Although collagen fibrillogenesis is a spontaneous entropy driven reaction, progress in the field of collagen research in the last decade has indicated that the process is under close cellular control. It is essential that mature fibrils fulfill the specific physical properties of different tissues: narrow (36 nm) fibrils are required in cornea where transparency is important, whereas large diameter (500 nm) fibrils are appropriate in mature tendon where high tensile strength is needed (Holmes *et al.* 2001; Meek and Fullwood 2001; Hulmes 2002). Furthermore, it is crucial for the cell's physiology that collagen fibrillogenesis progresses only at the appropriate time and in the appropriate place. The results presented in publication II indicated that α2β1 integrin expressed on fibroblasts and with the ability to bind to the fibrillar form of collagens would, among other cellular factors, participate in the regulation of collagen fibril formation. Indeed, type I collagen has been reported to have dozens of binding partners, which have been indicated in the regulation of the synthesis of highly organized collagen fibrils (Di Lullo *et al.* 2002).

The ability of the wild type α2I domain to inhibit fibrillogenesis and to bind tighter to collagen I monomers than to fibrils suggested that α2β1 integrins would first collect the newly synthesized collagen monomers close to the cell surface and then target fibril formation to the pericellular area. According to the model, newly formed fibrils would then be released and be again replaced with collagen monomers. To support the results obtained using soluble α2I domains, also a cell membrane bound α2β1 integrin heterodimer has been reported to be required for collagen I and III fibrillogenesis in fibroblasts derived from fibronectin -/- mice (Velling *et al.* 2002) and in vascular smooth muscle cells (Li *et al.* 2003b). Importantly α11β1 integrin, a small GTPase (RhoA) and fibronectin polymerization were also shown to participate in the process (Velling *et al.* 2002).

6.2.2. Both α2β1 and α11β1 integrin may be functional receptors for collagen fibrils

Collagen-binding integrins resemble each other. For example, the best characterized integrin high-affinity binding motif on collagen GFOGER, is recognized by all collagen receptor integrins: α1β1, α2β1, α10β1 and α11β1 (Emsley *et al.* 2000; Knight *et al.* 2000; Zhang *et al.* 2003; Gigout *et al.* 2008). Further, α2β1 integrins appeared not to be solely responsible for cell binding to collagen fibrils: human osteosarcoma cells (Saos, WT), which endogenously express only α1β1, α10β1 and α11β1 collagen receptor integrins, were able to mediate cell spreading on collagen fibrils. Functionally the α1 and α10 subunits are close to each other (Tulla *et al.* 2001). Since α1β1 integrin could not mediate cell spreading on collagen fibrils, α11β1 integrin is the most probable candidate for promoting the Saos cell spreading. To support this, the ligand binding preference of α11β1 integrin has been reported to resemble that of α2β1 integrin (Tiger *et al.* 2001). Additionally, it was recently suggested that α11β1 integrin has a role in collagen reorganization in myofibroblasts that concomitantly express α2β1 integrin (Carracedo *et al.* 2010). Consequently, both α2β1 and α11β1 integrins may mediate the essential interaction of cells with the surrounding collagen fibrils.

6.3. α2β1 INTEGRIN OUTSIDE-IN SIGNALING (II, III)

While study II established the role of α2β1 integrin as a receptor for the fibrillar form of collagen, further research is still required to clarify the details of α2β1 integrin outside-in signaling. The cell signaling induced by a monomeric and by a fibrillar collagen are thought to be different (Sato *et al.* 2003). The morphology of the spread Saos, CHO and HGF cells was analyzed in publication II. The results were consistent: cells formed wide lamellipodia on monomeric collagen, while fibrillar collagen induced the formation of long protusions. The formation of long cellular projections also characterizes the morphology of cells inside the collagen gel and in healing wounds. The collagen fibril induced cell signaling was not studied here, but small GTPases, the same molecules linked to α2β1 integrin-mediated signaling, have been previously implicated in the formation of cellular projections (Ivaska *et al.* 1999b; Banyard *et al.* 2000; Sato *et al.* 2003). Furthermore, one simplified example of modified α2β1 integrin signaling comes from studies using melanoma cells. In these cells, α2β1 integrin has been reported to mediate the regulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$. Shortly, $p27^{KIP1}$ appeared to be upregulated on fibrillar collagen and downregulated on monomeric collagen (Henriet *et al.* 2000). The upregulation of $p27^{KIP1}$ on fibrillar collagen was shown to lead to the inhibition of cell growth. It was suggested that the fibrillar form of collagen could not mediate α2β1 integrin clustering required for positive cell growth regulatory signal (Henriet *et al.* 2000). Based on the molecular modeling presented in this doctoral thesis, the collagen fibrils may, however, be able to cluster α2β1 integrins.

6.3.1. α2β1 integrin outside-in signaling relies on both conformational regulation and clustering

Ligand binding induced conformational modulation finally leads to the separation of the cytoplasmic and transmembrane domains of the integrin α and β subunits (Lu *et al.* 2001b; Kim *et al.* 2003; Luo *et al.* 2004; Nishida *et al.* 2006). The unclasped cytosolic domains further recruit signaling proteins to the forming focal adhesion site to initiate integrin outside-in signaling. In other words, integrin triggered signaling is initiated in response to ligand binding (Giancotti and Ruoslahti 1999; Schwartz and Ginsberg 2002). While receptor clustering and conformational regulation appeared to support each other in α2β1 integrin inside-out signaling, the mechanism of α2β1 integrin outside-in signaling might be different. The results presented in publication III indicate that α2β1 integrin outside-in signaling relies on both receptor clustering and conformational regulation.

Among other signaling proteins, α2β1 integrin has been linked to the heterodimer specific activation of p38 MAPK in different cell lines (Ivaska *et al.* 1999b; Ravanti *et al.* 1999; Xu *et al.* 2001; Bix *et al.* 2004; Mazharian *et al.* 2005). In publication III, α2 antibody-mediated clustering was shown to lead to rapid and transient p38 MAPK activation. Rapid α2β1 integrin internalization following antibody-generated clustering (Upla *et al.* 2004) could explain the transient nature of the p38 MAPK activation. In addition to clustering, similar allosteric conformational changes have been suggested to mediate both inside-out and outside-in signaling (Takagi *et al.* 2001; Takagi *et al.* 2002; Kim *et al.* 2003; Shimaoka *et al.* 2003). The mutation E336A in the α2 subunit prevents conformational changes in α2β1 integrin as described, however, the mutation does not prevent integrin cluster formation. However, the ability of CHO-α2E336A or Saos-α2E336A cells to form integrin clusters appeared not to be sufficient for p38 MAPK activation. Although the integrin αI domain can be considered flexible according to recent data, and the bent form of the integrin has been suggested to be also able to bind a large ligand (Xie *et al.* 2010), integrin signaling also requires the separation of the transmembrane and cytosolic parts of α and β subunits (Lu *et al.* 2001b; Kim *et al.* 2003; Luo *et al.* 2004; Nishida *et al.* 2006). The E336A mutation in α2 was shown to reduce, but not abolish, integrin-mediated cell adhesion to ligand. A lack of intersubunit communication may hold the integrin cytosolic domains clasped upon ligand binding. This may lead to the abolishment of α2β1 integrin outside-in signaling as described in publication III. The corresponding mutations in αL and αM integrins have been reported to abolish or markedly impair ligand binding (Alonso *et al.* 2002; Yang *et al.* 2004). Consequently, it may not be possible to perform a similar direct analysis of conformational requirements in outside-in signaling for those integrins.

6.4. α2β1 INTEGRIN AS A RECEPTOR FOR EV1 (III)

In addition to fibrillar collagens, EV1 binds to the α2I domain to initiate its life cycle (Bergelson *et al.* 1992; Bergelson *et al.* 1993). Also several other viruses have evolved to take advantage of cell surface integrins (Stewart and Nemerow 2007). Typically pathogens mimic the natural ligands of the receptor. However, EV1 has neither an RGD nor a typical integrin recognition sequence of collagens on its surface. Also other characteristic features of EV1 appear to differ from those of other viruses too: EV1 has been reported to accumulate in caveolin-1 positive structures and to be internalized through macropinocytosis (Karjalainen *et al.* 2008), while many other integrin-dependent viruses are internalized in clathrin-coated pits and found later in endosomes (Wickham *et al.* 1993; Joki-Korpela *et al.* 2001; Jin *et al.* 2002; O'Donnell *et al.* 2005). The unique behavior of EV1 stimulated the further exploration of the nature of the EV1 interaction with its cell surface receptor. The results are reported in publication III.

Integrin ligand binding, and the consequent conformational regulation, is based on the re-coordination of cations in the MIDAS (Michishita *et al.* 1993; Lee *et al.* 1995a; Emsley *et al.* 2000). However, EV1 binding is known to be a cation-independent process (Bergelson *et al.* 1993). Based on the comparison of the cryo-EM structure of EV1 in complex with the closed (Xing *et al.* 2004) and open α2I domain conformations, it could be concluded that most of the regions in α2I domain participating in the virus binding remain essentially unchanged during conformational alteration. Consequently, it remained unclear whether the virus would bind the inactive or active α2β1 integrin. However, the amino acid Asn289 in the αC helix of the α2I domain appeared to be in contact with EV1 only when the α2I domain was in the closed conformation suggesting that EV1 would favor the inactive conformation. Asn289 has been previously shown to be required for EV1 binding (Dickeson *et al.* 1999).

While EV1 binding to the α2I domain is a cation-independent process, and additionally cell surface integrins are suggested to stay in the low-affinity conformation in the absence of a cation (Xie *et al.* 2004), it was tempting to hypothesize that EV1 would recognize the bent α2β1 integrin. Based on the data from other integrins carrying a mutation that prevents intersubunit communication, also α2E336Aβ1 was suggested to stay in the bent conformation (Salas *et al.* 2004). As expected, the mutation E336A in the α2I domain strengthened EV1 adhesion to the domain. Similarly, a cation free environment enhanced EV1 binding to WT α2β1 integrin. Furthermore, the mutation E318W that leads to a constitutively active α2I domain and has been previously reported to increase α2I domain binding to collagen and decrease its ligand binding selectivity (Aquilina *et al.* 2002; Tulla *et al.* 2008), reduced EV1 binding as reported in publication III. Thus, experiments using recombinant α2I domains and cell lines harboring integrin mutations reveiled EV1 as the first integrin ligand reported to bind to the non-activated integrin. It has been previously hypothesized that viruses would compete with the natural ligands of integrins by having a stronger binding affinity to the receptor (Wang 2002). EV1 has been reported to bind to the α2I domain with about 10-fold higher affinity than

collagen I (Xing *et al.* 2004). A competitive mechanism may have evolved, since EV1 and collagen have been reported not to be able to bind to the α2I domain simultaneously (Xing *et al.* 2004). In addition to the stronger binding affinity, the ability of EV1 to recognize the low-affinity α2β1 integrin, which cannot be recognized by ECM ligands, might be beneficial for its entry.

As reported in publication III, conformational regulation is typically required for α2β1 integrin signaling following integrin adhesion to the ECM. However, all evidence supports the model in which EV1 has evolved to recognize the inactive integrin conformation. In agreement with this, others have recently reported that the bent form of integrin is able to bind a ligand (Bunch 2010). The model suggests that ligand binding itself would induce the extended integrin conformation. The possibility that EV1 induces a similar conformational change in the α2β1 integrin could not be excluded based on the structural model of the EV1 in complex with the α2I domain. However, the results indirectly revealed that conformational regulation would not be needed for EV1 entry: EV1 could not evoke p38 MAPK phosphorylation, which was shown here to require both α2β1 integrin clustering and an α2E336 –dependent conformational change. In contrast, the activation of PKCα appeared not to require integrin conformational regulation. Consequently, the α2E336A mutation did not prevent the PKCα-dependent (Upla *et al.* 2004) EV1 cell entry. Though the initial binding of virus to α2β1 integrin appeared not to induce p38 MAPK activation, p38 MAPK phosphorylation at the later stage of the EV1 infection has been detected by others (Huttunen *et al.* 1998). Since the p38 MAPK related signaling cascade is also known to be involved in several cellular processes including apoptosis (reviewed in Ono and Han 2000), p38 MAPK activation may be connected to EV1-induced cell lysis and the release of newly formed viral particles. The virus may have evolved to avoid the cell's defence system by acquiring the ability to enter the cell without inducing any cellular signaling mechanism at the early stage of infection. The results suggested that EV1 binding to α2β1 integrin would neither lead to conformational modulation in the receptor nor activate cellular signaling.

6.4.1. The bent form of α2β1 integrin is able to accommodate even a large ligand

It was previously assumed that in the resting cells, the ligand binding head domain stayed in a fixed position pointing towards the plasma membrane (Xiong *et al.* 2001). While the bent form of an integrin has been previously demonstrated to be able to bind a small ligand-mimetic RGD peptide (Xiong *et al.* 2002), the way the integrin accommodates the relatively large EV1 particle, with its icosahedral capsid of 30 nm in diameter (Filman *et al.* 1998) was not evident. Previously, other viruses have been shown to bind to the activated integrin. For example adenovirus type 12 has been seen to bind the extended conformation of the αVβ5 integrin ectodomain in a cryo-EM model (Lindert *et al.* 2009). The binding of five integrins to the viral capsid in either their extended or bent conformation may form different adhesion sites. The results presented in publication III support the model in which bent α2β1 integrins are bound at non-adjacent sites on the virus. Based on the model, integrin cytoplasmic domains would be located about 320 Å apart, possibly forming a unique ring-like adhesion site.

While the signaling proteins in a classical focal adhesion site have been intensively researched, the signaling proteins involved in EV1 entry still remain to be discovered.

The result presented in publication III indicated that the compact bent form of α2β1 integrin is not the physiologically inactive conformation, but is able to bind even large ligands. The first crystal structure of the complete ectodomain of an αI domain containing integrin, αXβ2, further supports the observation by demonstrating that the integrin αI domain is structurally more flexible than has been previously suggested and consequently able to bind a large ligand (Xie *et al.* 2010). Furthermore, a recently published model proposes that inside-out activation would cause the clustering of the bent integrins, and subsequent ligand binding would induce the extended integrin conformation (Bunch 2010). These findings together with the data from the publications presented in this thesis provide new insight into the regulation of integrin activation. Understanding the mechanism of α2β1 integrin activation could enable the discovery of new treatments for pathological conditions involving α2β1 integrin.

7. CONCLUSIONS

Like other integrins, α2β1 is not merely glue that mediates cell adhesion to the surrounding extracellular matrix. Instead it is involved in various biological and pathological conditions by transfering bidirectional signals across the cell membrane. α2β1 integrin signaling influences important biological events such as cell differentation, survival and apoptosis. The purpose of this thesis project was to study the requirements of α2β1 integrin conformational activation in inside-out and outside-in signaling. Furthermore, the structure-function relationships of α2β1 integrin interacting with the fibrillar form of collagen and EV1 were studied.

α2β1 integrin is present on the surface of several cell types and it is the most important integrin-type receptor for fibril forming collagens in their monomeric forms. In this thesis project, α2β1 integrin was shown to be a functional receptor also for collagen fibrils and was additionally suggested to participate in fibril formation. Furthermore, molecular modeling indicated that collagen fibrils are able to mediate integrin clustering.

The overall strength of integrin-mediated interactions between cells and the extracellular matrix is determined either by the conformational modulation of a single receptor or by the receptor clustering. In this thesis project, the conformational modulation of α2β1 integrin and receptor clustering were shown to strengthen the integrin inside-out activation in a synergistic manner. In contrast, ligand-induced α2β1 integrin signaling appeared to require both clustering and conformational modulation.

According to the conventional model, integrins in the bent conformation have been presumed to be inactive and incapable for ligand binding. In this conformation the ligand binding domain is pointing towards the cell membrane. Upon integrin activation the extracellular domain of the receptor has been suggested to straighten up. Based on the results of this thesis project, EV1 appeared to favor the bent α2β1 integrin conformation. The study demonstrated, for the first time, that the integrin in its bent form is able to bind a large ligand. Recently, the analysis of crystal structures showed that the αI domain is more flexible than was previously proposed. The model, together with the results from this study suggests that the distinction between the inactive and the active integrin is faltering. The detailed mechanisms of the αI domain containing integrin activation still remain to be solved. The formation of focal adhesion sites and the subsequent cellular signaling may also vary depending on the conformation of the integrin upon ligand binding.

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