Role of evolutionary conserved residues in integrin α11 cytoplasmic tail

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Abstract

Integrins are cell surface receptors, present in the plasma membrane of cells. The integrin family is composed of 18 α subunits and 8 β subunits, which can combine non-covalently to form 24 different integrin heterodimers. Integrin α11β1 is the major collagen-binding integrin on fibroblasts and is involved in myofibroblast differentiation, wound healing and stroma-regulated effects on tumorigenesis. The integrin α11 cytoplasmic tail is suggested to have a role in integrin α11β1 function, but the relative role of individual amino acids in α11 cytoplasmic tail is still unclear. Sequence analysis of α11 cytoplasmic tails revealed that arginine-1174 and lysine-1185 are evolutionary conserved, suggesting that these conserved amino acids contribute to integrin α11 function. To elucidate the potential role of these conserved amino acids in the α11 cytoplasmic tail, we mutated the conserved arginine-R1174 and lysine-K1185 to alanines. α11 cDNAs encoding mutant integrin chains, wildtype α11 and cytoplasmic tail deleted α11, were all tagged with EGFP and virally infected and stably expressed in C2C12 mouse myoblasts which lack endogenous collagen receptors. The contribution of the conserved arginine-1174 and lysine-1185 was analyzed in cell adhesion-, focal adhesion formation-, proliferation- and migration assays. Our data demonstrate that the K1185A mutation affected focal adhesion formation, reduced cell proliferation and cell migration. In contrast, the R1174A mutation did not have any significant effect in these assays, except for mediating focal adhesion formation. In summary, our results suggest that conserved lysine-1185 of integrin α11 cytoplasmic tail is essential for α11 integrin function.

Contents

Acronyms and Abbreviations

APS	Ammonium Persulfate
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
DAPI	4', 6-Diamidino-2-Phenylindole
DEL	Deletion
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DOK-1	Docking Protein 1
DT	Distal Mutation
ECL	Enhanced Chemiluminiscence
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
FA	Focal Adhesion
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FERM	Four-point-one protein, Ezrin, Radixin, Moesin
FLNA	Filamin-A
FLNB	Filamin-B
FLNC	Filamin-C
GAP	GTPase-Activating Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFFKR	Glycine-Phenylalanine-Phenylalanine-Lysine-Arginine
GFFRS	Glycine-Phenylalanine-Phenylalanine-Arginine-Serine
GFOGER	Glycine-phenylalanine-Hydroxyproline-Glycine-Glutamic acid-Arginine
HDR(R/K)E	Histidine- Aspartic acid - Arginine(Arginine/Lysine)- Glutamic acid
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HRP	Horseradish Peroxidase
IAC	Integrin Adhesion Complex

ICAP-1	Integrin Cytoplasmic Domain-Associated Protein-1
IgG	Immunoglobulin G
ILK	Integrin-Linked Kinase
IMC	Inner Membrane Clasp
ITGA11	Human Integrin α11 Gene
JNK	c-Jun N-Terminal Kinase
KO	Knock Out
LAD III	Leukocyte-Adhesion Deficiency Type III
LDV	Leucine-Aspartic acid-Valine
LUBAC	Linear Ubiquitin Chain Assembly Complex
NA	Nascent Adhesion
NPxY/F	Asparagine-Proline-any amino acid-Tyrosine/Phenylalanine
OMC	Outer Membrane Clasp
ON	Over Night
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PH	Pleckstrin Homology
PIP2	Phosphatidyl Inositol 4,5-Bisphosphate
PSI	Plexin-Semaphorin-Integrin
PT	Proximal Mutation
PTB	Phosphotyrosine-Binding (Domain)
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic Acid
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFK	Src Family Kinases
SHANK	SH3 and Multiple Ankyrin Repeat Domains Protein
SHARPIN	SHANK-Associated RH Domain-Interacting Protein
TBS-T	Tris-Buffered Saline with Tween-20
TEMED	N, N, N′, N′-Tetramethylethylenediamine
TGF-β	Transforming Growth Factor Beta
TIRF	Total Internal Reflection Fluorescence
WT	Wild Type

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1 Introduction

1.1 The extracellular matrix

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs that provides vital physical scaffolding for the cellular constituents and induces crucial biochemical and biomechanical cues required for cell differentiation, tissue morphogenesis, and tissue homeostasis. The ECM also acts as a storage depot for growth factors and cytokines (1). It comprises of magnificent diversity of molecules and is composed of collagens, proteoglycans, and a large number of multiadhesive glycoproteins (2).

Collagens are the most abundant protein in vertebrates that are composed of collagen triple helices. The collagen family has 28 members (collagen types I -XXVIII) which are the product of 49 distinct collagen α-chain genes (3). Among them, type I collagen, is the major collagen in the vertebrate body and gives strength and flexibility to tissues such as bone, tendons, ligaments, and skin. In addition, collagen I forms macromolecular structures for embedding of resident mesenchymal cells. Thus, cell-extracellular matrix interactions are significant not only for preserving tissue properties but also for the phenotype of resident cells (4). Collagens are broadly categorized into fibrillar and non-fibrillar types but some collagen types can also assemble into a variety of supramolecular structrues including reticular networks and sheets. The organization, distribution, and density of fibrils, networks and the additional supramolecular structures formed, vary with collagen- and tissue types (3).

1.2 Integrins

Cell adhesion is essential for tissue formation and integrity (2). Integrins constitute a central family of cell adhesive receptors that mediate cell-ECM and cell-cell interactions. In the late 80s, integral membrane protein complexes were identified from a number of sources and named 'integrins' to denote their integrating role and importance for the integrity of the ECM to cytoskeleton linkage. Integrins also take part in specialized tranisent cell-cell interactions with membrane protein ligands. The integrin family is composed of 24 αβ heterodimeric members which are assembled from 18α and 8β subunits (**Figure 1**) (5, 6).
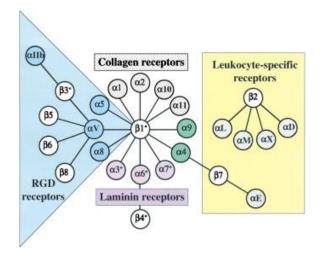

Figure 1: The integrin family *including subclasses according to their ligands, figure from Hynes et al (7).*

Integrin interactions induce changes in cytoskeletal organization, protein phosphorylation, and gene expression (8, 9). Integrin signaling is essential for normal cellular processes such as: cell migration, cell growth, cell survival, and cell differentiation which are involved in a variety of physiological process like embryonic development, tissue homeostasis and maintenance, and wound healing. Integrins also play important roles in tumor progression and metastasis (10, 11). Integrin α and β subunits are non-covalently associated to form heterodimers. Each subunit is composed of different domains with flexible linkers connecting them. Both subunits have a single membrane-spanning helix which form the transmembrane domain, and a cytoplasmic tail, which is usually short. The size of the two subunit varies from integrin to integrin, but commonly, the α- and β-subunits contain around 1000 and 750 amino acids, respectively (6).

The extracellular part of integrin α subunits has four or five extracellular domains that form a seven-bladed β-propeller, a thigh domain, two calf domains, named calf-1 and calf-2 (**Figure 2**). Additionally, a domain called the inserted (I) domain is present in 9 out of 18 α subunits. Four of these integrins with αI domains are collagen receptors, namely α1β1, α2β1, α10β1, and α11β1. The αI domain comprises nearly 190 amino acids and is situated between blades 2 and 3 of the β-propeller. This domain can undergo conformational changes that are important for regulating the binding affinity. The essential parts for inter-domain flexibility are the linker regions between the β-propeller and the thigh domain and "genu" or knee at the bend between the thigh and the calf-1 domain (6).
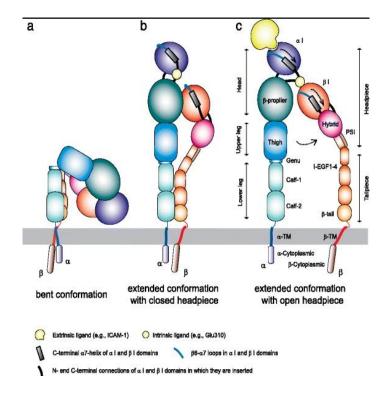

Figure 2: Schematic illustration of integrin domains. *a. Bent conformation containing the closed headpiece (with low-affinity I domain). b. Extended conformation but having the closed headpiece (with intermediate-affinity I-domain). c. Extended conformation containing the open headpiece (with the high-affinity I domain). Figure from Park et al (12).*

The extracellular part of the β subunit is composed of seven domains with flexible interconnections. These domains include the βI domain which is homologous to the αI domain, a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, four cysteine-rich epidermal growth factor (EGF) modules, and a β tail domain. Like the α subunit, the β subunit also contains 'knee', situated between the EGF1 and EGF2 domains (6, 13).

1.2.1   Cytoplasmic tails of integrin subunits

The cytoplasmic tails of integrin subunits are commonly not extending more than 75 amino acids long (except the β4 tail, which has approximately 1,000 amino acids). There is remarkable homology among the β-subunit cytoplasmic tails, while the α-subunit cytoplasmic tails are significantly diverging except for a conserved GFFKR motif in proximity to the transmembrane region (**Figure 3**). Many cytoskeletal and signaling proteins have been shown to bind to β cytoplasmic tails, and some have also been found to associate with α tails. The cytoplasmic tails of integrins are conformationally flexible; they can form either α-helices or β-strand, depending on their interacting molecules. One or two conserved NPxY/F motifs are common in integrin β tails, where phosphorylation of the tyrosine (Y) can regulate integrin interactions with other proteins at the cytosolic site of the plasma membrane (13-15).

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αL     KVGFFKRNLKEKMEAGRGVPNGIPAEDSEQLASGQEAGDPGGCLKPLHEKDSES[1140]GGGKD
αM     KLGFFKRQYKDMMS[1126]EGGPPGAEPQ
αX     KVGFFKRQYKEMMEEANGQIAPENGTQTPS[1158]PPSEK
αD     KLGFFKRHYKEMLEDKPEDTATFSGDDFSCVAPNVPLS
αV     RMGFFKRVRPPQEEQEREQLQPHENGEGNSET
αE     KCGFFKRKYQQLNLESIRKAQLKSENLLEEEN
α1     KIGFFKRPLKKKMEK
α2     KLGFFKRKYEKMTKNPDEIDETTELSS
αIIb   KVGFFKRNRPPLEEDDEEGE
α3     KCGFFKRARTRALYEAKRQKAEMKSQPSETERLTDDY
α4     KAGFFKRQYKSILQEENRRDS[988]WSYINSKSNDD
α5     KLGFFKRSLPYGTAMEKAQLKPPATSDA
α6     KCGFFKRNKKDHYDATYHKAEIHAQPSDKERLTSDA
α7     KCGFFHRSSQSSSFPTNYHRACLAVQPSAMEGGPGTVGWSSNGSTPRPPCPSTMR
α8     KCGFFDRARPPQEDMTDREQLTNDKTPEA
α9     KMGFFRRRYKEIIEAEKNRKENEDSWDWVQKNQ
α10    KLGFFAHKKIPEEKREEKLEQ
α11    KLGFFRSARRRREPGLDPTPKVLE
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β2     KALIHLSDLREY[735]RRFEKEKLKS[745]QWNNDNPLFKS[756]AT[758]TTVMNPKFAFES
β3     KLLITIHDRKEFAKFEEERARAKWDTANNPLY[747]KEATST[753]FTNITY[759]RGT
β5     KLLVTIHDRREFAKQSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD
β6     KLLSSFHDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC
β7     RLSVEIYDRREYSRFEKEQQQLNWKQDSNPLYKSAIT[782]TTINPRFQEADSPTL
β8     RQVILQWNSNKIKSSSDYRVSASKKDKLILQSVCTRAVTYRREKPREIKDISKLNAHETFRCNF
```

Figure 3: Integrin cytoplasmic tail sequences. *Alignment of the human α and β integrin chains shows the conserved membrane-proximal sequences in α chains and functionally important NPxY sequences, as well as the phosphorylation sites of the α and β chains (denoted with amino acid numbers); from Gahmberg et al (16).*

1.3 Integrin ligands

Although integrins have a wide variety of ligands, integrin-ligand combinations can be classified into four main classes, depending on the molecular interaction's structural basis. Eight out of twenty-four integrins, can recognize ligands containing an RGD sequence such as fibronectin, vitronectin and fibrinogen. Integrin $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$ bind to an acidic motif called LDV sequence that is functionally related to RGD sequence. Four α subunits, all with an αI domain ($\alpha 1$, $\alpha 2$, $\alpha 10$, and $\alpha 11$) together with $\beta 1$ are collagen receptors and bind to the GFOGER-like sequence in triple helical collagen (17-19). The combination of $\alpha 3$, $\alpha 6$, and $\alpha 7$ chains with $\beta 1$, and $\alpha 6$ with $\beta 4$, are highly selective laminin receptors (**Table 1**). The four members of the the $\beta 2$ subfamily bind to ligands recognized by immune cells. Additional integrin ligands also exist (20).

ECM protein	ECM sequence	Integrins
Collagens	GFOGER	$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$
Fibronectin	RGD	$\alpha 5\beta 1$, $\alpha V\beta 3$, $\alpha 8\beta 1$, $\alpha V\beta 1$, $\alpha V\beta 6$, $\alpha IIb\beta 3$
Laminins	LDV	$\alpha 4\beta 1$, $\alpha 4\beta 7$
	REDV	$\alpha 4\beta 1$
	E1´ fragment	$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$
	E8 fragment(SIEKP)	$\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 6\beta 4$
Vitronectin	RGD	$\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha V\beta 1$, $\alpha V\beta 5$, $\alpha V\beta 8$
Fibrinogen	RGD	$\alpha V\beta 3$
	KQAGDV	$\alpha IIb\beta 3$
Von Willebrand factor	RGD	$\alpha IIb\beta 3$, $\alpha V\beta 3$

Table 1. *List of adhesive sequences in ECM proteins and their corresponding integrin receptors. Compiled from Barczyk et al (21).*

1.4 Integrin activation

Inactive, active, and active with ligand-occupied: these are three states of integrins (22).

Integrin activation is a must-needed process, where bent (inactive) integrin is changed to extended form (active) by conformational changes required for integrin signaling for other responses, cell motility, and gene expression. This activation can be done in bidirectional way, either 'outside-in' or 'inside-out' signaling (23, 24). Talin, a cytoskeletal protein, acts as an indispensable mediator of inside-out signaling. Talins binds to proximal NPxY motif of β cytoplasmic tails via phosphotyrosine binding domain (PTB)-like domain and this interrupts a salt bridge between α and β cytoplasmic tails, causing tail separation and integrin activation. Moreover, Kindlins, another family of cytoskeletal protein which bind to the distal NPxY motif of integrin β tails, are also essential regulators for integrin activation. Kindlins have a negligible effect without talins and can only synergize with talins to activate and cluster integrins (24-26).

Integrins become activated by experiencing conformational changes controlled by inside-out signals, which in turn triggers outside-in signals. In outside-in signaling, after ligand binding,

integrin undergoes several conformational changes that break both outer membrane clasps (OMC) and inner membrane clasps (IMC) by which both tails become separated. These conformational changes contribute to integrin activation (25, 27, 28).

Integrin activation requires controlled temporal and spatial regulation for proper functioning. It is now realized that besides direct integrin activators, there are proteins that can suppress integrin activation by binding either α- or β-tails. Some proteins like filamin-A, DOK1 (Docking protein 1), ICAP-1 (integrin cytoplasmic domain–associated protein-1) bind with NPxY motifs of β cytoplasmic tails, which overlap with the talin- and kindlin-binding sites, thus suppressing integrin activation (**Figure 4**) (15). Additionally, SHARPIN (SHANK-associated RH domain-interacting protein) is an integrin inhibitor which by binding to the highly conserved WKxGFFKR sequence present in the membrane proximal part of integrin α chain cytoplasmic tails inhibits talin-mediated integrin activation (29).
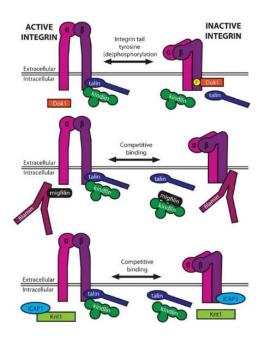

Figure 4: Regulation of integrin activation and inactivation. *Figure from Morse et al 2014(15).*

Talins

Talins are cytoplasmic adaptor proteins. Vertebrates have two main talin isoforms, called talin 1 and talin 2. Talin 1 has a molecular weight of 270 kDa, and can bind with the cytoplasmic domain of integrin β subunits, which causes integrin activation (30, 31). It contains a globular N-terminal head region and a flexible rod domain. The head comprises a FERM (F for 4.1 protein, E for Ezrin, R for Radixin, M for Moesin) domain and there are binding sites for the cytoplasmic domain of β-integrin, layilin, filamentous actin (F-actin), PIPK1γ90 [a splice variant of phosphatidylinositol (4)-phosphate 5-kinase type Iγ] and focal adhesion kinase (FAK). The talin rod domain also contains a binding site for integrin cytoplasmic tails. This domain can also bind to actin and vinculin (32, 33). A free, intact talin resides in an auto-inhibited 'closed' conformation in the resting state. Talin remains precisely self-masked by attaching the β-tail-binding region of talin-FERM (F3 domain) domain with its rod domain. This inhibition can be disrupted by talin activator phosphatidylinositol 4, 5-bisphosphate (PIP2) by the "pull-push" model. Studies showed that PIP2

shows the most potent binding compared to other lipids present on the inner surface of the plasma membrane (33).

Kindlins

Like talin, kindlins also belong to the FERM-containing protein family and have an essential role in integrin activation. Kindlins -1, -2, -3 constitute the three members of the kindlin family in vertebrates, each containing a FERM domain and a PH (Pleckstrin homology) domain. The kindlin-FERM domain is insignificantly exceptional by having an additional N-terminal F0 lobe from the typical FERM domain-containing three lobes (F1, F2, and F3) (34). Kindlin-1 is expressed in epithelia, and nonfunctional kindlin-1 mutations result in a congenital skin disease called Kindler syndrome (34, 35). Kindlin-2 is widely expressed, and kindlin-2 null mice are characterized by peri-implantation lethality (36). Expression of kindlin-3 is confined to the hematopoietic system, and kindlin-3 defects have been associated with leukocyte-adhesion deficiency type III in humans (LAD III) (37). Kindlins are also involved in tumorigenesis and metastasis (34).

Filamin A

The filamin family contain actin-binding proteins and consists of three homologous proteins, called Filamin-A (FLNA), Filamin-B (FLNB), and Filamin-C (FLNC). Filamin-A is encoded by the X-linked gene *FLNA*. Filamin-A is liable for cross-linking actin filaments to make orthogonal networks. It also connects actin networks to the plasma membrane to maintain the characteristic shape and motility of the cell. In addition, filamin-A can act as a scaffold protein for signaling molecules that mediate intracellular protein trafficking (38). Because of its diverse function in regulating cell motility and signaling, defects in the FLNA gene cause a wide range of developmental anomalies involving the brain, bone, limbs, and heart (38, 39). Filamin competes with talin for binding to an overlapping site (distal NPxY motif)) in the integrin β cytoplasmic tail. Studies in multiple cell lines have shown that absence or decreased expression of filamin can enhance integrin-mediated cell-substrate adhesion (40).

DOK1

Dok proteins are members of the family of adaptors which are phosphorylated by different protein tyrosine kinase (PTKs). The ideal member of the family is DOK-1 (molecular weight of 62 kDa), which when associated with Ras GTPase-activating protein (RasGAP) acts as a negative regulator of Ras (41). DOK- 1 can also bind with the same membrane-proximal NPxY motif in integrin β cytoplasmic tails as talin and compete with talin, resulting in integrin inactivation (29).

SHARPIN

SHARPIN is a cytosolic protein with a relative molecular weight of 40 kDa which was first identified in the postsynaptic density of excitatory synapses in the brain, where it binds SHANK (SH3 and multiple Ankyrin repeat domains protein) proteins (42). Later studies showed that SHARPIN represses integrin activity in many cell types, and SHARPIN null mice display increased β1-activity. While other integrin inhibitors directly compete with talin or kindlin by

binding with integrin β cytoplasmic tails, SHARPIN binds with proximal conserved residues of α subunit cytoplasmic tails and maintains integrin inactivation by stabilizing the proximal salt bridge between integrin α/β tails (43). SHARPIN also functions as a subunit of LUBAC, which stimulates linear ubiquitin chain assembly of proteins involved in cell signaling (43, 44).

1.5   Integrin-mediated cell adhesion

Cell adhesion mediates interactions with the ECM and neighboring cells and is central for transferring biochemical and biophysical information from the cell exterior to the cell inside. Integrin-mediated adhesion begins following integrin activation. After ligand binding, integrins generate the assembly of different adhesion structures that vary in their morphology, mechanical properties, and also protein composition (45, 46). The first adhesion structures, called nascent adhesions (NA). They are short-lived, near the edge of cell protrusions, and their size is around 100 nm. Many NAs disassemble, while some of them grow into bigger focal adhesions (FA). FA maturation is a coordinated process demanding integrin clustering, fibrous actin bundling, and consolidation of the linkages connecting integrin and actomyosin (45, 47, 48). The interactions between vinculin, talin and kindlin cause maturation of FAs (49). As FA maturation is considered a tension-dependent process, several studies have shown the significance of myosin II cross-linking and radial stress fiber assembly in FA growth (50-52). Once FAs have formed, they can either disassemble or form fibrillar adhesions. For example, in the case of α5β1, FAs are transported to the cell center and converted to fibrillar adhesions. Fibrillar adhesions are elongated structures rich in tensin-1 and kank-2 in which the actin cytoskeleton and fibronectin are connected through integrin α5β1 (53-57).

1.6   Cell migration

Cell migration plays the central role in normal development, wound healing, immunological responses, and cancer metastasis (58, 59). The migration process requires complex mechanical communications between cells and the underlying substrate (60). For initiation of migration, cells can undergo a multiplied series of events, like protrusions, adhesion formation, and stabilization at the leading edge; after that, cell body translocation, the release of adhesions, and finally detachment of the posterior part of the cells which causes cells to move forward (61).

For mediating cell migration, integrin-containing adhesions arrange a network of signaling pathways. The Rho GTPases serve as a merging point for these networks and regulate actin polymerization, dynamics, and adhesion. In addition, adhesions signaling can be localized and thereby stimulate the polarized events that comprise migration (61).

Integrin-based adhesions that induce cell migration are FAs; sub classified as NA, focal complexes, FAs as described earlier, podosomes and invadopodia. The NA employ strong propulsive traction to drive cell migration, but consequently, this force decreases as the FAs mature into large plaques. Finally, most of the mature FAs are localized centrally in a migrating cell, and, in spite of having their persistent substrate adhesion, only resistive forces are exerted against forwarding migration (60, 61).

1.6.1 Spheroids as a tool to investigate cellular migration

In vitro cells are cultured in a monolayer which is quite different from the unique environment of cells *in vivo*. Because, in the natural environment, cells remain in compact arrangements, where they are surrounded by ECM that allows them to communicate with adjacent cells and ECM, particularly for managing specific cellular function and growth. Two-dimensional monolayer cultures can obtain a homogenous cellular environment that allow studies of several cellular processes. However, this is not the natural environment for cells. To obtain a more *in vivo*-like conditions , cells can be cultured in three-dimensional culture systems, one of which is spheroid model (62, 63). An embedded spheroids system facilitates studies of physiological cellular cell-ECM interactions, metabolism, growth, and invasion. Collagen is the most widespread embedding material in spheroid studies, and collagen stiffness or pore size can be modified during preparation of the matrix. In cultured embedded spheroids one frequently can observe two cell populations, a migratory population and a non-migratory core where cell-matrix interactions control the migratory population and cell-cell contacts manage the non-migratory core (64).

1.7 Integrin signaling

Integrin signaling is essential for integrin mediated effects during cell migration, cell survival, cell differentiation, and cell motility. Integrins do not, unlike most growth factor receptors, possess endogenous enzymatic activity. In order to activate downstream signaling pathways, integrins can recruit multiple proteins to form integrin 'adhesome' of interacting proteins, some of which have enzymatic activity (65). The integrin adhesion complex (IAC) is a multimolecular structure that forms by the association of integrins and the actin cytoskeleton, and a network of approximately 60 to 100 proteins first identified by mass spectrometry (66, 67). The integrin-mediated signaling leads to phosphorylation of proteins within the IAC and activation of downstream signaling pathways (68).

1.7.1 FAK and Src signaling

The focal adhesion kinase (FAK), a core component of IAC, is a tyrosine kinase and is one of the earliest recruited IAC components (69, 70). FAK maintains cell migration and IAC dynamics. The rate of cell spreading and migration goes down in FAK-null cells (71, 72). Following integrin binding, FAK indirectly associated with integrins and becomes activated by autophosphorylation at its tyrosine 397 (Y^{397}). Y^{397} is an auto phosphorylation site, and a high-affinity binding site for Src homology 2 (SH2) domains of the Src family kinases (72, 73). Src-dependent phosphorylation of FAK at Y^{576} and Y^{577} occurs after Src recruitment, which further increases adhesion-induced FAK activation (68). The FAK-Src signaling complex can activate Rho GTPases. Rho GTPases are central regulators of the cytoskeleton dynamics during cell spreading and migration (74, 75).

1.8 Role of integrin cytoplasmic tails

Integrin cytoplasmic tails are short in contrast with the extracellular part of integrins, but these small regions are essential for integrin function (8, 15).

Integrins cytoplasmic tails form cytoskeletal linkages and affect multiple signaling pathways (13, 65).

These communications are strongly regulated by many circumstances, such as separation of cytoplasmic tails, post-translational modifications in the tails, and competitive interference of proteins binding to similar sites in the cytoplasmic tails (15, 76).

As already mentioned, integrin α-tails contain a conserved membrane-proximal GFFXR core motif; the rest of the tails have unique α chain specific-sequences which is in contrast to integrin β-tails, which contain multiple conserved amino acids sequence motifs in their cytoplasmic tails (**Figure 3**) (15).

Integrins are maintained in a resting stage by a salt bridge between the arginine (R) of the GFFKR sequence in the α tail and aspartate (D) of the conserved HDR(R/K)E motif in the β cytoplasmic tail. This salt bridge can be disrupted by a point mutation of the arginine, which causes integrin activation to occur (77, 78). Most β-tails have two NPxY motifs, and as discussed above, many proteins compete for binding to these sites.

As also mentioned in previous sections, tyrosine phosphorylation of the NPxY motifs occurs by Src family kinases (SFK). After phosphorylation, integrin activation is regulated positively or negatively by binding PTB domain-containing proteins, like talin and Dok1(15). However, integrin tails can also affect in integrin recycling and turnover. Integrin trafficking is controled by Rab21, a small GTPase that interacts with the GFFXX domain of the integrin α cytoplasmic tails (79).

1.9   Collagen-binding integrins

Among the 24 integrin family, there is a subgroup of collagen-binding αI domain-containing integrins. This subgroup includes α1β1, α2β1, α10β1, and α11β1. In addition, the α chain cytoplasmic tails are thought to contribute to diverse intracellular signaling (80). Analysis of unchallenged knockout (KO) mice have revealed a mild phenotype during their development. More recently, analyses of mice lacking integrin α11β1 have revealed crucial roles during wound healing, fibrosis, and tumor-stroma interactions (19, 81, 82).

1.9.1   Integrin α11β1

1.9.1.1   Structure of integrin α11

Integrin α11β1 is the last identified member of the integrin family in vertebrates. It is the primary collagen-binding receptor on fibroblasts, and like other collagen-binding integrins, it also contains αI domain. Human integrin α11 subunit is encoded by the human α11 integrin gene (*ITGA11*), which is found on chromosome 15q23. The human α11 subunit is composed of 1188 amino acids and this subunit contains 22 inserted amino acids in the extracellular region (amino acids 804–826) of unknown function. Immunoprecipitation with antibodies to α11 integrin captures a protein that migrates at 145-kDa in nonreducing conditions and 155-kDa under reducing conditions in SDS-PAGE (83-86).

1.9.1.2   Expression of integrin α11

The expression of integrin α11 was first identified in cultured human fetal muscle cells in vitro Later studies have confirmed α11 expression in subsets of fibroblasts around ribs, vertebrae, intervertebral discs, and keratocytes of the cornea of 8-week human embryos (83, 84). Moreover, α11 expression is also detected in tumor tissue from various solid tumors from patients, including breast, pancreas, and lung (19, 87-89). Furthermore, integrin α11 expression seems significant

during myofibroblast differentiation and cancer-associated fibroblasts (CAFs), suggesting that α11 also has a potential role in pathological conditions related to fibroblast functions in fibrosis and tumor progression. In addition, integrin α11 affects tumor stroma stiffness and promotes tumorigenesis in non-small cell lung cancer models (10, 90).

1.9.1.3 Functions of integrin α11β1 in vivo

In the developing mouse head, integrin α11 is restricted to the ectomesenchymal cells, including the periodontal ligament. Studies of RNA and protein in developing mouse embryos have verified a mesenchymal expression (91). Studies showed that α11-deficient mice are viable and fertile but have an incisor eruption phenotype causing malnutrition and increased mortality (82-84). Moreover, integrin α11-null mice show a lack of granulation tissue formation and decreased JNK activation by non-canonical TGF-β signaling in an excisional wound healing model in mouse skin (10).

1.9.1.4 Functions of integrin α11β1 in vitro

Integrin α11β1 can mediate adhesion of cells, support FA formation, and stimulate cell migration on collagen I in culture (91, 92). In addition, it provides cells with the ability to contract fibrillar collagen gels, which corresponds to collagen reorganization mediated by myofibroblasts during development, wound repair, and fibrosis (92). A recent study showed integrin α11 cytoplasmic tail involvement in integrin α11β1 functions like FA formation, cell proliferation, cell migration, and collagen remodeling (81). This study demonstrates that cells expressing tail-less human α11 (Huα11-1171) had fewer FA with reduction of the total area of FA by 50%, compared to cells having full-length Huα11. Not only in FA, truncated α11 expressing cells also displayed reduction in collagen gel contraction and cell migration than WT Huα11 expressing cells. This study also clarified that the cytoplasmic tail of integrin α11 involved FAK and ERK activation, while the α11 tail did not affect p38 and JNK activation (81). Although the role of α11 cytoplasmic tail has been confirmed in deletion experiments (81), the relative role of individual amino acids is still unclear. Therefore, in this study, we use a mutational analysis to look at the functions of conserved amino acids in the α11 cytoplasmic tail.

2 Objectives

2.1 Main Objectives

The overall aim of the study is to further understand the role of integrin α11 cytoplasmic tail.

2.2 Specific Objectives

1. To investigate the role of conserved residues for integrin α11 cytoplasmic tail in focal adhesion formation.

2. To investigate the role of conserved residues for integrin α11 cytoplasmic tail in cell proliferation.

3. To investigate the role of conserved residues for integrin α11 cytoplasmic tail in cell migration.

3 Materials and methods

3.1 Materials

3.1.1 Cell lines

Cell line	Cell type	Origin	Source
C2C12	Myoblast	Mouse	ATCC

3.1.2 Plasmid

Plasmid	source
pBabe-puro Retroviral vector	Addgene Plasmid #1764

3.1.3 Materials for cell culture

Materials	Supplier
Dulbecco's modified Eagle medium (DMEM)	Gibco; cat#31966-021
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma; cat# D8537
Fetal Bovine Serum (FBS)	Gibco; cat#10270-106
Antibiotic antimycotic (AntiAnti)	Gibco; cat#15240-062
0.05% Trypsin- EDTA	Gibco; cat#25300-054
Casyton	OLS, cat#5651808
Dimethyl Sulfoxide (DMSO)	Sigma; cat# D2650
Puromycin	Gibco, cat#A11138-03
Cell culture flasks 75 cm^2 (T75)	Nunc
CryoTubeTM vials	Nunc; cat#375418
Coverslip $\varnothing$ 14mm	MatTek, cat#P354
Cell strainer-40 um	Fisher brand; cat#22363547
Multi-well plate (24, 48,96 well plate)	Nunc

3.1.4   Materials for experiments

Materials	Supplier, cat/ref number
Acrylamide 37.5-30%	BIO-RAD, cat# 1610158
Ammonia persulfate (APS)	Sigma-Aldrich Inc, cat# A3678
Bovine serum albumin (BSA)	Roche; cat# 10735094001
Collagen I	PureCol; cat# 5005
Crystal violet	Sigma; cat#V5265
DAPI solution	Invitrogen, ref# P36935
ECL Western Blotting Detection Kit	Thermo scientific, cat#32106
Fibronectin	Sigma-Aldrich;cat#F0895-2MG
Paraformaldehyde	Thermo scientific; cat#28908
Magic Marker XP protein standard	Invitrogen, ref# P/N LC5602
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma Aldrich, cat# T9281
Precision plus protein standards (Dual color)	Bio-Rad; cat# 161-0374
Sodium Dodecyl Sulfate (SDS)	VWR International; cat# 44215HN
Trizma® Base	Sigma, cat#t1503
Triton X-100	Sigma, cat# 9002-93-1
Tween-20	Sigma Aldrich, cat# P 2287
Methylcellulose	Sigma Aldrich, cat no- MO512-100G
2x DMEM	Merk Millipore, cat# SLM-202-B
4X Sample buffer	Bio-Rad, cat# 1610791
0.2M HEPES	Sigma Aldrich, cat # H3375

3.1.5 Primary antibodies

Antibodies	Host	Supplier
Anti-human integrin α11; clone 24	Mouse	Nanotools, custom-made
Anti-GAPDH	Mouse	Santa Cruz Biotechnology, cat# sc-32233
anti-human integrin α11, clone 6	Mouse	Nanotools, custom-made

3.1.6 Secondary antibodies

Antibodies	Host	supplier
Alexafluor® 647 anti-mouse IgG	Goat	Jackson ImmunoResearch cat# 115-605-003
Alexafluor® 594 anti-mouse IgG	Goat	Jackson ImmunoResearch, cat # 115-585-003
HRP conjugated anti-mouse IgG antibody	Goat	Santa Cruz Biotechnology cat# sc2005

3.1.7 Reagents for 7.5% Polyacrylamide gel

3.1.7.1 Running gel (20 ml)

Materials	Amount
Acrylamide/Bis (37.5:1, 30%; BioRad)	5ml
Tris-HCl 2M pH8.8	4 ml
H_2O	11 ml
SDS 20%	100 µl
TEMED	20 µl
APS 10%	100 µl

3.1.7.2 Stacking gel (6ml):

Materials	Amount
Acrylamide/Bis (37.5:1, 30%; BioRad)	1ml
Tris-HCl 0.5M pH 6.8	1 ml
H_2O	4 ml
SDS 20%	30 µl
TEMED	20 µl
APS 10%	30 µl

3.1.8   Instruments and softwares

Instrument and software	Company
Casy ModelII TT cell counter	Schärfe System
Cell Profiler software	Broad Institute
ChemiDoc^{TM} XRS	Bio-Rad Laboratories Inc
BD Accuri C6 flow cytometer	BD, Biosciences
Fiji Image J software	Image J
FLOWJO software	BD, Biosciences
GraphPad Prism software version 9	GraphPad
Hera cell 150i (Heraeus) incubator	Thermofisher
HeraSafe laminar flow bench	Kendro
iXon 888 Life EMCCD camera	Andor - Oxford Instruments
Nikon Ti-E inverted microscope	Andor - Oxford Instruments
Quantity One software	Bio-Rad Laboratories Inc
SoftMax Pro software	Molecular Devices
SONY CELL SORTER	Sony Biotechnology
VERSAmax Microplate reader	Molecular Devices
Zeiss fluorescence microscope	Carl Zeiss

3.2 Methods

3.2.1 Generation of cell lines

Four cell lines have been used in the project. The cells lines had previously been generated by transducing the mouse myoblast cell line C2C12 (ATCC, cat#CRL-1772) with the cDNA encoding variant of integrin α11 with EGFP tag in the pBABE-puro viral vector (Addgene) (81). Thus, four different cell lines were generated, expressing wild-type (WT) integrin α11 subunit (with intact cytoplasmic tail of α11), α11 subunit with proximal mutation (PT) or distal mutation (DT) in cytoplasmic tail, or α11 subunit with deletion (DEL) of cytoplasmic tail after GFFRS motif, respectively (the mutation and deletion sites of the cytoplasmic tail are indicated in a schematic map in Figure-6). Constructs and transductions were previously performed by a former PhD student in Gullberg's lab and was available for this study.

3.2.2 Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, cat#31966-021) with 10% fetal bovine serum (FBS; Gibco; cat#10270-106) and 1% antibiotic-antimycotic (Anti-anti, Gibco; cat#15240-062). The cells were routinely handled in a clean lamina flow hood to secure sterility and inspected under Carl Zeiss Primovert microscope to observe confluency. They were cultured in a 75 cm^2 T75 flask up to 80-90% confluence before performing experiments. All the cultures were kept at a Hera cell 150i (Heraeus) incubator with a humidified atmosphere of 5% CO_2 in air at 37°C.

3.2.2.1 Thawing of the cells

Complete culture medium (DMEM with 10% FBS and 1% anti-anti) was preheated in a 37°C water bath before the cryo-preserved cells (see details in section 3.2.2.4) were taken out from the liquid nitrogen-tank. The vials were heated in a 37°C water bath for a couple of minutes for thawing the cells and vials were then transferred to the laminar air hood. T75 culture flasks were prepared with 12 ml of preheated culture medium and labeled after the cells were transferred from the corresponding cryo-vial. The medium was replaced with fresh medium after two hours. The cells were kept growing in the Hera cell 150i incubator until they reach 80% confluency.

3.2.2.2   Passaging of the cells:

The cells were routinely passaged every 2 to 3 days when the culture reached 80-90% confluence. A vacuum pump was used to remove the old medium from the culture flasks, and the cells were gently washed with Dulbecco's Phosphate Buffered Saline (PBS; Sigma; cat# D8537). 1ml 0.05% Trypsin-EDTA (Gibco; cat#25300-054) was added to each flask and incubated for around one min to allow trypsin to break down the proteins that help the cells to adhere to the culture flasks and gently tapped on the side of the flasks for complete detachment. Complete culture medium was then added to the flask to stop trypsinization and cells were resuspended to get homogenized single-cell suspension. For routine passaging, the cells were split in a ratio of 1 to 10 - 1/10 of the cell suspension was left in the flask to continue the cell culture. The rest of the cell suspensions were either discarded or transferred to 15 ml tubes for further experiments.

3.2.2.3   Cell counting:

We need to count cells in every experiment as we need the same number of cells to compare results between the different cell lines. We used the Casy Model II TT cell counter for the procedure. 50 µl cell suspension was taken from 10 ml cell suspension, mixed with 10 ml Casyton (OLS, ct#5651808).After that, the cell counter analyzed 10 ml Casyton with 50 µl cell suspension; Upon activation, the machine counted the total, live, dead cell, and viability percentage of cells.

3.2.2.4   Freezing of cells

For long-term storage, cells require to be frozen and preserved in liquid nitrogen. First, freezing media was prepared with 10% Dimethyl Sulfoxide (DMSO; Sigma; Cat no. D2650), 20% FBS, and 70% DMEM and kept on ice. After reaching 70-90% confluency, cells were washed with PBS, trypsinized (as previously described in 3.2.2.2), resuspended and centrifuged at 1000 rpm (310 g) for 5 min. Afterwards, the supernatant was discarded, the pellet was resuspended in freezing medium, and 1 ml of each cell-freezing medium suspension was carefully aliquoted into 1.8 ml CryoTubeTM vials (cat#375418, Nunc). The vials were labeled with cell line names, passage number, date, and the name of the person who froze the cells. Then put the vials into a Corning CoolCell freezing box to control the freezing rate to −1°C/minute when placed in a −80°C freezer. The following day, the cells were transferred from the -80°C freezer to a liquid nitrogen tank for long-term storage.

3.2.3 Fluorescence-activated cell sorting (FACS)

FACS was performed to enrich the cell populations with similar expression levels of α11 variants in the cell lines, the four cell lines were cultured until sub-confluent, trypsinized, and resuspended in DMEM with 10%FBS. Cells were then washed twice with plain DMEM. Following the last wash, cells were resuspended in PBS with 2% FBS and cell suspensions were filtered through 40 µm Fisherbrand Sterile Cell Strainers (Fisher scientific; cat#22363547) to eliminate cell clumps. Around 2×10^6 cells were used for sorting against EGFP intensity in SONY CELL SORTER. The C2C12 parental cells (without fluorescence) were also prepared as a negative control to set up the sorting gates. 2×10^5 sorted cells were collected and centrifuged at 310 g (1000 rpm) for 5 min, and cell pellets were resuspended in the culture medium with puromycin at a final concentration of 5 µg/ml (Gibco, cat#A11138-03) and cultured up to sub-confluent.

3.2.4 SDS-PAGE (Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis)

3.2.4.1 Gel preparation

20 ml of running gel solution was made by mixing the reagent mentioned in 3.1.7.1. After adding TEMED and 10% APS, the gel solution was poured in a gel preparation cassette from BIO-RAD, leaving around 2 cm below the comb's bottom for the stacking gel. The running gel was polymerized after 45 - 50 min, then 6 ml of stacking gel was made by mixing the chemicals mentioned in 3.1.7.2, which was then poured on top of the running gel and use a comb for making ten wells. After 20 min the gel was ready to use.

3.2.4.2 Sample preparation

Subconfluent cells were trypsinized (see 3.2.2.2), resuspended in DMEM with 10%FBS, and counted. After that 3×10^6 cells were centrifuged at 1000 rpm for 5 min. The pellets were dissolved in 150 ul of 1x sample buffer (diluted from 4x Laemmli sample buffer, Bio-Rad, cat#1610747). After sonication at 4°C, the samples were centrifuged at maximum speed (13,000 rmp) for 10 min on a bench centrifuge (Eppendorf) and the supernatant were collected. The samples should be boiled at 95°C for 5 min before they were used for SDS-PAGE. Unused samples can be stored at -20°C for later experiments.

3.2.4.3 Protein separation

The prepared SDS-polyacrylamide gels were set into the Bio-Rad tank filled with 1x running buffer (diluted from 10x Tris/Glycine/SDS buffer, Bio-Rad cat# 1610772). Samples were then loaded into the wells of the gels (35 μl /well, around 15-20 μg protein/well). Precision plus protein standards (10 to 250 kDa from Bio-Rad) and MagicMark XP (Novex, Cat# LC5603) were mixed 1:1 and loaded 6 μl/well in 2 wells. The gel was run for approximately 1 hour at 95V.

3.2.4.4 Western-blotting (Transfer of protein and protein immunoblotting)

Following separation on SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane by the iBlot dry transfer system (Invitrogen). After 7 min transferring, the excess membrane was cut and washed briefly with Tris-buffered solution with 0.05% Tween-20 (TBS-T). 5% solution of non-fat dry milk in TBS-T was used for blocking the membrane for 1 hour at RT. After blocking, the membrane was incubated with primary antibodies, mouse anti-GAPDH (Santa Cruz Biotechnology, cat# sc-32233; 1:1000), and mouse monoclonal anti-human α11 IgG, clone24 (Nanotools, cat#1:50) in TBS-T at 4°c for ON. The following day the membrane was washed in TBS-T for three times-10 min each. Then the membrane was incubated with secondary antibody HRP conjugated goat anti-mouse antibody (1:5000) for 1 hour at RT, before final wash in TBS-T. We used the 1% solution of non-fat dry milk in TBS-T for dilution of antibodies. The membrane was developed using ECL Western Blotting Detection Kit (Thermo Scientific, cat#32106) and photographed using ChemiDoc and Quantity One analysis software (Bio-Rad). Finally, band densities were quantified using Image J software, and relative expression levels were calculated.

3.2.5 Analysis of cell surface protein expression by flow cytometry

Flow cytometry was performed to know the cell surface expression levels of α11 variants of the cell lines. After trypsinization of the sub confluent cells (see 3.2.2.2), the cell suspensions were collected in the 15 ml tube and washed twice with PBS. 2×10^6 cells were prepared for each sample which were blocked with 1ml of 2% Bovine serum albumin (BSA, Roche; cat# 10735094001) in PBS, at RT for 30 min. Then, 1×10^6 cells were pipetted in the 15ml tube and

centrifuged for 5 min at 310 g. After blocking, cells were incubated with primary antibody, mouse monoclonal anti human α11 antibody (Nanotools, 210f4b6a4' clone 24) at 1: 250 in 2% BSA in PBS at 37°C (water bath) for 1 hour. Afterwards, cells were washed twice with PBS and incubated with secondary antibody, Goat anti-mouse IgG conjugated with Alexa 647 at 1:500 in 2%BSA in PBS at RT for 1hr. Cells were then washed twice with PBS and analyzed using BD Accuri C6 flow cytometer for the intensity of Alexa 647 using the same gating for all samples. We used FLOWJO software to analyze the data from Accuri.

3.2.6   Cell attachment assay

We started the experiment by coating a 48-well culture plate with either collagen I (10 µg/ml; PureCol; cat# 5005), fibronectin (2 µg/ml; Sigma-Aldrich; cat#F0895-2MG), or 2% BSA in PBS for 2 hours at 37°C. After two hours, the wells were washed with PBS once and blocked with 2% BSA in PBS for 1 hour at 37°C. During blocking time, cells were trypsinized and washed with DMEM thrice. Cell were then counted and 1×10^5 cells per well were seeded in the well in 250 µl DMEM. Following incubating the cells for 50 min at 37°C, unattached cells were removed by gently washing twice with PBS containing $Ca^{2+}$and Mg^{2+}. Afterwards, the cells were fixed with 96% ethanol for 10 min at RT, washed with PBS containing $Ca^{2+}$and Mg^{2+} once, and then stained with 0.1% crystal violet solution (Sigma; V5265) for 20 min at RT. After staining, the cells were washed with distilled water until the excess color was removed, lysed using 1% Triton X-100 (Sigma, cat#9002-93-1) for 25 min and cell lysates were transfer to a 96-well plate. The absorbance of the cell lysate was read at 596 nm in an ELISA reader.

3.2.7   Focal adhesion assay

We used ∅35 mm petri dishes specially designed for TIRF microscopy where a ∅14mm coverslip is glued to the middle of the dish (MatTek, cat#P354), coated the coverslips with Collagen type I (100 µg/ml in cold PBS), 500 µl/plate at 37°C for 1 hour. Afterwards, the plates were washed twice with PBS. Cells were trypsinized, resuspended in DMEM with 10%FBS, counted, and seeded 4×10^5 cells/well onto the coverslips for 2 hours at 37°C. After two hours, fixed the cells were fixed with 4% PFA (freshly prepared from a 16% PFA solution, Thermo scientific; cat#28908) for 10 min at RT, then washed them with 1xPBS twice for 5 min each. Then blocked the cells were blocked with 5% BSA/0.1% Triton X-100 in PBS for 1 hour,

flowed by incubating the cells with the primary antibody, mouse monoclonal anti-human α11 IgG, clone-6 from nanoTools (1:400), for 1 hour at 37°C. After 1 hour, cells were washed with PBS/0.05% Tween-20 thrice, 10 min each. Secondary antibody goat anti-mouse, Alexa 594(1:500) was then applied and incubated for 1 hour at RT. Then cells were washed for 30 minutes again with PBS/0.05% Tween-20 like before, stained with DAPI solution (0.25 μg/ml in PBS) for up to 1 min, then washed with PBS for 2×5 min. FA images were captured using inverted TIRF microscope. We used Dragonfly 505 (Andor Technologies, Inc) system, which was equipped with an inverted Nikon Ti-E microscope, and took image using an iXon 888 Life EMCCD camera. After taking pictures we analyzed the data with Cell Profiler software.

3.2.8   Cell proliferation assay

1×10^5 Cells were seeded per well on a 24-well plate which was pre-coated with collagen I (10 μg/ml; PureCol; cat# 5005) and fibronectin (2 μg/ml; Sigma-Aldrich; cat# F0895-2MG) for 2 hours at 37°C. The cells were cultured for 24 hours with DMEM containing 1% FBS at 37°C in the incubator. The following day, media and unattached cells were removed by gentlely washing twice with PBS containing Ca2+and Mg2+ and cells were fixed using 96% ethanol for 10 minutes at RT. Then cells were stained with 0.1% crystal violet (Crystal Violet Solution; Sigma; V5265) for 20 min at RT and washed with distilled water until the excess color was removed. Then the cells were lysed using 1% Triton X-100(Sigma, cat#9002-93-1) for 25 min and cell lysates were transfer to a 96-well plate, and absorbance of cell lysate in each well was read at 596nm in an ELISA reader.

3.2.9   Cell migration assay in 3D collagen gel

Homospheroids were made for the four cell lines (WT, PT, DT, Del) using the hanging drop method. Briefly, subconfluent cells were trypsinized, counted, and resuspended in culture medium with 20% Methylcellulose solution to get a cell suspension of 1×10^6 cells/ml. Around 40 drops of the cell suspension (20 μl/drop; 2×10^4 cells/drop) were pipetted on a lid of a non-treated Petri dish (100 mm). The lid was then carefully inverted over the Petri dish containing DMEM, and the cells in drops were cultured at 37°C in the incubator overnight to form spheroids.

Next day, the spheroids were ready for mixing with collagen I solution for the migration assay. Collagen I solution was made by mixing the following reagents: five parts of 2x DMEM (Merk Millipore, cat# SLM-202-B,), four parts of collagen I (10 µg/ml; PureCol; cat# 5005), and one part of 0.2M HEPES (Sigma Aldrich, cat # H3375) at pH 8, and then 100 µl per well of the collagen gel solution was added into the 96 well plate. After 20 min when the gel was semi polymerized, spheroids were embedded into the gel, one spheroid per gel. Then the collagen gel with spheroid was allowed to polymerize at 37°c in the incubator for one hour. After polymerizing, 100 µl of DMEM with 4% FBS was added to each well and cultured for 24 hours. Spheroids were visualized under Zeiss fluorescence microscope, and pictures were taken at 0, 2, 4, 6, 8, and 24 hours.

4   Results

4.1   Identification of conserved residues in the cytoplasmic tail of integrin α11

Evolutionary conserved residues in integrin cytoplasmic tails are suggested to have a functional role in integrin function (93). To identify the key amino acid residues that are important for integrin α11 cytoplasmic tail function, we first compared the amino acid sequences of integrin α11 between different species for identifying conserved amino acid residues in the cytoplasmic tail of integrin α11. We performed sequence alignment for the cytoplasmic tail sequence of integrin α11 across human, mouse and zebrafish using Clustal Omega, a web-based sequence alignment program (94). Sequence alignment shows that arginine (R) 1174 and lysine (K) 1185 in the cytoplasmic tail of α11 are conserved among human, mouse, and zebrafish in addition to the membrane proximal GFF sequence, which is conserved in all integrin α-tails (Figure 5). To this end, we hypothesized that the evolutionary conserved arginine-1174 and lysine-1185 could have an essential role in integrin α11 function.

Origin	Gene	Aminoacid Sequence of α11 Cytoplasmic Tail
Human	ITGA11	GFFRSARR - RREPGLDPTPKVLE
Mouse	Itga11	GFFKSAKR - KREPGLGPIPKELK
Zebrafish	itga11a	GFFQRQKRREEA -ENEVNGKTMEER
Zebrafish	itga11b	GFFHRQKRKRDE -- QEANEKTAEDR

Figure 5: Conserved residues in the cytoplasmic tail of α11 in different species

4.2   Generation of cell lines expressing integrin α11 variants

To investigate the importance of R1174 and K1185 in integrin α11 function, the coding sequence for the two conserved residues were either mutated or deleted as shown in Figure 6. All α11 variants were tagged with enhanced green fluorescent protein (EGFP) for better visualization and stably expressed in C2C12 mouse myoblasts by retroviral transduction based on pBABE viral vector as described in Erusappan et al (81) . The C2C12 cells were used because these cells lack endogenous expression of collagen-binding integrins, which make the cell line a "clean" system to study the function of α11 integrin. Four cell lines expressing different human α11 integrin variants were generated: α11 integrin with full-length cytoplasmic tail (Hu α11-WT), α11 with mutation in cytoplasmic tail R1174A (proximal conserved amino acid mutation, Hu α11-PT), α11 with mutation in K1185A (distal conserved amino acid mutation, Hu α11-DT), and α11 with deletion of 17 terminal amino acids of cytoplasmic tail after the GFFRS motif (Hu α11-DEL).
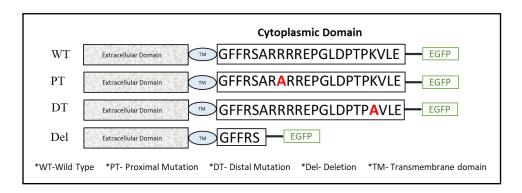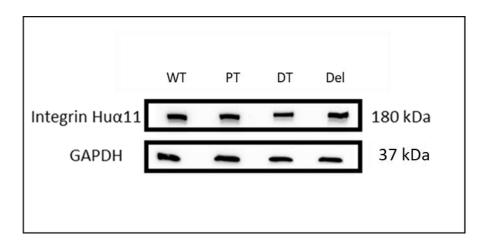
Figure 6: Schematic map of the four constructs encoding α11 integrin variants tagged with EGFP. *The WT construct has intact cytoplasmic tail, while in PT arginine-1174 was mutated to Alanine, and in DT lysine-1185 was mutated to Alanine. In Del construct, the C-terminal amino acids of cytoplasmic tail were deleted after GFFRS motif.*

4.3   C2C12 cell lines expressing different α11 variants display comparable total and cell surface protein levels of integrin α11

Inorder to compare α11 function, it is imperative to have similar α11 expression levels in all the four C2C12 cell ines expressing α11 variants. To achieve equivalent expression levels, all the cell lines were sorted against EGFP by fluorescence-activated cell sorting (FACS) with the same gating. After growing and expanding the sorted cells, the expression level of the α11 variants (tagged with EGFP) in each cell line were analyzed by Western blotting. Densitometry analysis of integrin α11 bands shows that total protein levels of integrin α11 are similar without any significant difference between the different C2C12 cell lines expressing α11 variants (Figure7A and 7B).
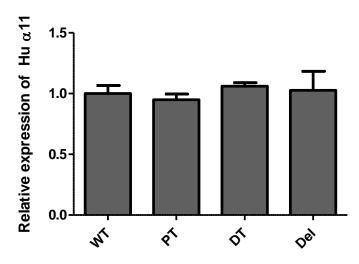
A.

B.

Figure 7: Comparison of expression levels of the α11 integrin variants between the cell lines by Western Blotting. *A. Representative immunoblot showing total protein expression of α11 integrin (EGFP-tagged) in C2C12 cells expressing α11variants. B. Graph showing relative expression of Hu α11 normalized to GAPDH from at least three independent experiments repeated at different passages.*

We next examined the cell surface expression of α11 to see if the amount of α11 expressed at the cell surface is comparable between the different C2C12 cell lines expressing α11 variants. We analyzed cell surface expression of α11 by FACS after staining with integrin α11 monoclonal antibody. Untransfected C2C12 cell line was used as a negative control. FACS data shows similar cell surface expression levels of α11 between the different C2C12 cell lines expressing α11 variants (Figure 8).
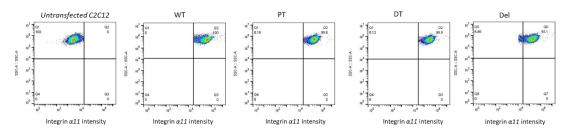

Figure 8: FACS analysis *showing cell surface expression of α11 in C2C12 cells expressing α11 variants or Untransfected C2C12 cells.*

4.4 Point mutation on R1174 or K1185 of integrin α11 cytoplasmic tail does not influence α11-mediated cell adhesion to collagen

Charged residues in the membrane proximal regions for some integrin α-cytoplasmic tails have been shown to have a role in cell adhesion by influencing integrin activation (95). To investigate the role of R1174 and K1185 in integrin activation, we compared the cell adhesion function of the cells expressing integrin α11 variants on collagen. Fibronectin or BSA coating were also used as positive and negative controls, respectively. Results show similar cell adhesion for both PT

and DT cells in comparison with WT cells. These results suggest that point mutation on R1174 or K1185 of integrin α11 cytoplasmic tail does not influence α11- mediated cell adhesion.
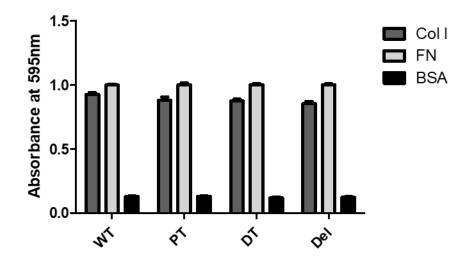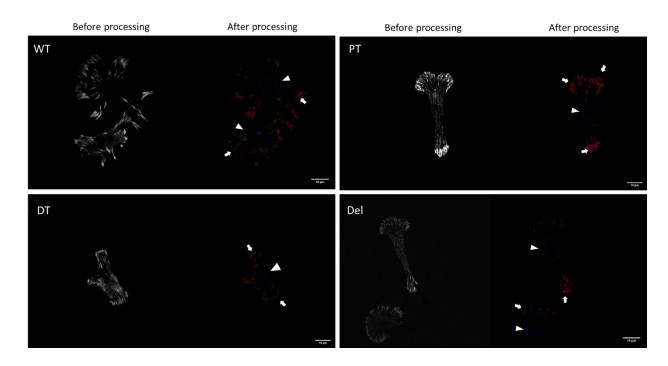

Figure 9: Cell attachment of Hu α11 integrin on collagen I; *Bar chart showing values from cell attachment experiment, where each bar presents the average value pooled from three independent experiments done in triplicates and the error bars represent standard deviation.*

4.5   Point mutation on K1185 of integrin α11 cytoplasmic tail affects integrin α11-mediated focal adhesion maturation on collagen matrices

Terminal 17 amino acids in the cytoplasmic tail of integrin α11 was suggested to have a role in mediating FAs formation (81) . Therefore, we asked if the conserved R1174 and K1185 could have a role in α11-mediated FA formation. In this context, we compared the FAs of cells expressing different integrin α11 variants on collagen. FAs were imaged using TIRF microscopy and automated image analysis were performed using Cell Profiler software. Interestingly, both DT and PT cells had a significant reduction in the amount of FAs compared to WT (Figure 10A-B). However, DT cells had a stronger reduction in FAs and was similar to DEL cells. These results indicate that point mutation on R1174 or K1185 α11 cytoplasmic tail affects α11-mediated FA formation.
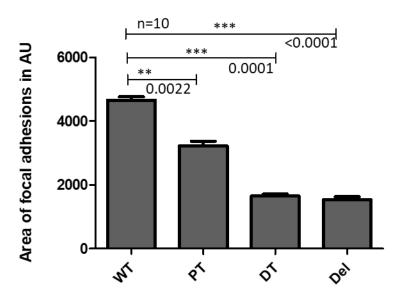
A.

B.

Figure 10: Formation of FA by integrin α11. *A. Pictures showing presence of integrin α11-FAs (arrows) and nucleus (arrowhead). Scale bar: 10 μm. B. Bar chart showing values from FA quantification (n=10 cells), where each bar presents the average value from three repetitive experiments and the error bars represent standard deviation.* Statistical significance was assessed by two tailed, unpaired t-tests and P-values are expressed as ***, P<0.001, **, P<0.01.

4.6   Point mutation on conserved K1185 of integrin α11 cytoplasmic tail affects α11-mediated cell proliferation

FAs are central for cell adhesion mediated intracellular signaling which are essential for cell proliferation. Since, both PT and DT cells had an effect on FA, we hypothesized that R1174 and 1185 could have a role in cell proliferation (96). To investigate the role of R1174 and K1185 in cell proliferation, all the cell lines expressing α11 were cultured in collagen I or fibronectin-coated wells with low serum concentration for 24 hours. The results show that cell proliferation was reduced for DT cells in comparison with WT cells on collagen and the defect in cell proliferation for DT cells were similar to DEL cells. PT cells had no significant difference in cell proliferation when compared with WT cells. The cell proliferation results indicate that point mutation K1185 of integrin α11 cytoplasmic tail could have role in regulating α11-mediated cell proliferation.
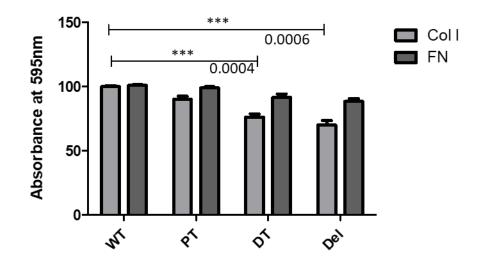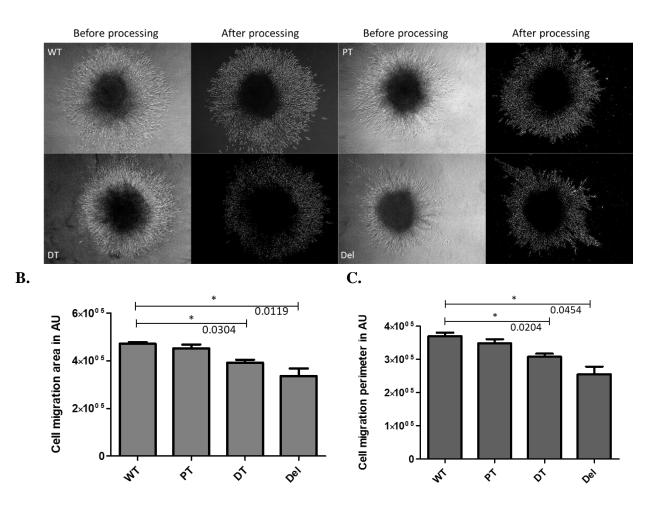

Figure 11: Role of integrin α11 variants in cell proliferation. Graph showing absorbance values for cell proliferation on collagen I and fibronectin. Each bar presents the average value pooled from three independent experiments done in triplicates. B. normalize value of cell lines compared to WT for collagen I. Statistical significance was assessed by two tailed, unpaired t-tests and P-values are expressed as ***, P<0.001.

4.7   Point mutation on conservedK1185 of integrin α11 cytoplasmic tail affects α11-mediated 3D spheroid cell migration

Based on our data on cell proliferation and FA, we asked if R1174 or K1185 of integrin α11 cytoplasmic tail could have role in cell migration (97). To determine the effect of the conserved R1174 and K1185 of integrin a11 in cell migration, homospheroids composed of WT, PT, DT, or Del cells were embedded in 3D collagen I gel. Migration of the cells from spheroids were imaged after 24 hour and processed through Cell profiler software with an automated fashion to measure

cell migration from the spheroid (Figure 12A). Cell migration area and perimeter were used to assess spheroid cell migration. The results show that cell migration was impaired in DT cells in comparison to the WT cells (Figure 12B). However, the reduction in cell migration for DT cells were not pronounced as in DEL cells and PT cells did not have any significant effect on cell migration when compare to WT

A.

B.

C.

Figure 12: Spheroid migration assay *A. Pictures showing representative images of spheroids of original and processed outlines of migratory cells. B. Graph showing cell migration area. C. Graph showing perimeter of cell migration. Results shown are pooled data from three independent experiments done with at least 3 spheroids per experiment. Statistical significance was assessed by two tailed, unpaired t-tests and P-values are expressed as *, P<0.05.*

5   Discussion

Integrin α11β1 is a major collagen receptor for fibroblasts and its cytoplasmic tail has been suggested to have central role in the signaling function (81) . However, the molecular details on how the integrin α11 cytoplasmic tail is contributing to integrin α11β1 signaling remains unclear. In this study, we aim to characterize the role of conserved amino acids in the α11 cytoplasmic domain in order to understand the function of integrin α11 cytoplasmic tail.

Mutational analyses of conserved amino acids are well established to investigate integrin cytoplasmic tail interactions (26, 78). To characterize the α11 cytoplasmic tail functions, we adopted mutational analyses on conserved residues as a strategy. Therefore, we characterized the effect of point mutation on the conserved R1184 and K1185 of α11 cytoplasmic tail in integrin α11β1 function using cell-based functional assays.

In our study, we made point mutations (R1174A, K1185A) or deletion mutation (Δ1172 -1188) and expressed these α11 variants as C-terminal EGFP fusion proteins in C2C12 mouse myoblasts. Our cell attachment data show that EGFP fusion did not affect integrin function in mediating cell adhesion similar to studies overexpressing EGFP-tagged integrin α5 in CHO cells (98).

Our results from the cell attachment assay show that the expressed human α11 variants were functional and mediates cell adhesion of C2C12 to collagen. Membrane-proximal arginine in the conserved GFFKR motifs in α tails for some integrins contribute in salt bridge formation with β tails to inactivate or to maintain integrin at inactivated states (99). Point mutation of arginine in GFFKR motif of αL, αIIb, and α4 caused salt-bridge disruption and activated integrin in a ligand-independent manner (78) (81). Furthermore, membrane-proximal GFFKR was shown to interact with SHARPIN affecting integrin activation (43). Since α11-tail lacks GFFKR, we hypothesized if the conserved R1174 could have role in integrin inactivation. However, our results suggest that neither R1174 nor K1185 are involved in influencing integrin active or inactive conformations in these conditions and need better models to validate this aspect.

FA formation, maturation and stabilization or turnover are central aspects for integrin signaling. Terminal 17 residues in the human cytoplasmic tail of α11 are suggested to contribute for α11-mediated FA formation. In this study, we characterized the role of conserved R1174 and K1185 in FA formation. Our results show that both R1174 and K1185 are involved in α11-mediated FA formation since substitution of these residues by alanine affected the ability of α11 in localizing to FAs. Particularly, K1185A had a stronger effect on FA and was equivalent to the cells expressing α11 variant without the terminal 17 residues in the α11-tail. These data suggest that K1185 in the membrane distal region of α11-tail could have a potential role in α11β1-mediated FA formation. Membrane distal region of integrin α-tails are suggested to strengthen integrin β-tail interactions with kindlin, a cytoskeleton protein that regulates integrin function (100).

As described in earlier sections, cell adhesion-mediated intracellular signaling is essential for cell proliferation, migration and survival. Integrin α11-tail is suggested to regulate α11-mediated cell

proliferation and migration. We therefore investigated the role of R1174 and K1185 in cell proliferation and migration. Our cell proliferation results indicate that K1185 could contribute for α11-mediated cell proliferation. Based on the findings from FAs, we speculate that the role of K1185 could be a possible explanation for reduced cell proliferation. Defect in cell adhesion signaling has been demonstrated to affect cell proliferation (69).

Finally, we characterized the role of R1174 and K1185 of α11 cytoplasmic tail in cell migration. Similar to the proliferation results, our spheroid migration data suggests that K1185 of α11 cytoplasmic tail could have a role in regulating α11-mediated cell migration. The role of K1185 in FA formation could be a possible explanation for the defect in cell migration. In addition, there is also a possibility that K1185 could influence cell migration by regulating integrin turnover. Terminal lysines in the cytoplasmic tail of integrin α tails are suggested to be involved in integrin turnover through ubiquitination and point mutation in the lysine residues of integrin α5-tail affected integrin α5 turnover and cell migration on fibronectin (101, 102). Since K1185 is the only lysine residue in human integrin α11-tail, we speculate that K1185 could also have an additional role in regulating integrin α11 turnover as mechanism for controlling cell migration, besides its role in FAs.

In summary, our results suggest that conserved lysine-1185 is essential for integrin α11β1 function as it is involved in mediating FA formation, cell proliferation, and migration.

6 Conclusion

In this mutational study, we try to establish the importance of evolutionary conserved amino acids of integrin α11 cytoplasmic tail in integrin α11β1 function. Our data show that, although both mutations are involved in α11 mediated FA formation, the significance of K1185 is prominent. In addition, our results undoubtedly confirm that K1185 contributes to cell proliferation and migration, whereas the mechanism for this is not clear.

7 Future perspectives

Our study demonstrates that conserved K1185 of α11 tail has an essential role in α11β1 activation. However, there are still some questions to understand the role of K1185 in integrin α11β1 function. As point mutation of K1185 causes defective FA formation, we could hypothesize that K1185 contributes to cell adhesion signaling. So, it would be interesting to investigate the role of K1185 in cell adhesion signaling. Besides, it is also important to investigate the possible protein interactions for the α11 cytoplasmic tail and the role of K1185 on this context. Also, to detect the reason for defective cell migration because of K1185, we could investigate the role of K1185 in regulating integrin α11 turnover.

8 References

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