Extracellular Matrix Receptors in Astrogliosis

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Abbreviations

AChR	Acethylcholine receptor
CNS	Central nervous system
CRIB	The Cdc42 binding domain of PAK
CSPGs	Chondroitin sulphate proteoglycans
DG	Dystroglycan
DGC	Dystrophin glycoprotein complex
ECM	Extracellular matrix
FN	Fibronectin
GAP	GTPase Activating Proteins
GDI	Guanosine nucleotide dissociation inhibitors
GEF	Guanine exchange protein
Glu MT	Detyrosinated, stable microtubule
GM1	Ganglioside; component of lipid rafts
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
LN	Laminin
LPA	Lysophosphatidic acid
MT	Microtubule
MTOC	Microtubule organizing center
PAK	p21-activated kinase
RGD	arginine-glycine-aspartic acid peptide sequence
RT	Room temperature (22-23 ^{o}C)

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Résumé

Dystroglycan et les intégrines sont deux types de récepteurs de la matrice extracellulaire (RMEC) impliqués dans l'adhésion et la migration des astrocytes. Les mécanismes moléculaires qui contrôlent leur distribution aux plates-formes de signalisation de la membrane plasmique demeurent peu étudiés. Dans la présente étude, nous définissons quatre stages d'adhésion cellulaire pour les astrocytes dans lesquels DG et les intégrines sont distribués différemment. Dans la cellule en migration, les deux récepteurs sont recrutés à l'avant, possiblement dans des îlots riches en GM1. En combinant la microscopie à fluorescence et l'usage de perturbateurs du cytosquelette dans notre modèle de lésion *in vitro*, nous démontrons aussi que la formation des protrusions riches en microtubules n'est pas actine-indépendante. Cependant, la formation d'actine corticale est indépendante du réseau polarisé de microtubules. Nous confirmons l'activation rapide de Cdc42 et Rac1 suite à une lésion *in vitro* et avons développé une lignée d'astrocytes DG-nulles pour étudier le rôle de DG dans l'activation des Rho GTPases. Nous concluons que la distribution des RMEC est étroitement régulée avec l'appareil de réorganisation cytoskelettique dans la prolifération astrocytaire.

Abstract

Dystroglycan and the integrins are two types of extracellular matrix receptors involved in astrocyte adhesion and migration. The molecular mechanisms that control their distribution to signaling platforms of the plasma membrane remain poorly understood. In the present study, we define four cell spreading stages for adherent astrocytes, termed stage 1 to 4, and defined by the state of spreading of the cell in culture. Immunocytochemistry analysis reveals that DG and integrins distribute differentially depending on the stage, and both receptors get recruited to leading edge of the cell, possibly to GM1-rich lipid rafts, in scratch-induced migration. Treatment of reactive astrocytes with cytochalasin-D or colchicine confirms that the formation of MT-rich protrusions is not actin-independent. However, cortical actin localization is independent of the polarized MT network. We confirm scratch-induced Cdc42/Rac1 activation early after wounding and further investigated the role of DG in Rho GTPases signaling. We conclude that ECM receptors localization is tightly regulated along with the cytoskeletal reorganization machinery in astrocyte proliferation.

Chapter 1- Introduction

Dystroglycan is a ubiquitously expressed, extracellular matrix (ECM) receptor encoded by a single gene (*dag1*), and synthesized as a precursor protein that is cleaved into α and β subunits. This heterodimer protein is an essential component of the dystrophin-glycoprotein complex and has been studied intensively in skeletal muscle, where it is thought to play a role in maintaining the structural integrity of the sarcolemma and in clustering acetylcholine receptors at the neuromuscular junction (Jacobson *et al*, 1998; Montanaro *et al*, 1999; Heathcote et al, 2000). The N-terminal peripheral membrane protein α-dystroglycan (α–DG), which is highly glycosylated in its mature form, binds directly to the ECM molecules laminin, perlecan, agrin and neurexin through its O-linked carbohydrates. Biglycan is another ligand for α-DG but the interaction does not require glycosylation of α-DG (Bowe et al, 2000). α-DG is noncovalently attached to the N-terminal domain of the transmembrane β-dystroglycan (β-DG) which links α-DG to the actin cytoskeleton via dystrophin or its autosomal homologue, utrophin. β-DG is known to associate directly to a variety of cytoplasmic molecules involved in cell signaling such as caveolin-3, Grb2 and Erk sig suggesting a role for dystroglycan as a scaffold for adhesion-dependent signal transduction (Spence *et al*, 2004; Langenbach and Rando, 2002).

A growing body of evidence suggests a role for dystroglycan in cell polarity and cell adhesion for a number of different cell types. For example, Drosophila dystroglycan is required cell-autonomously for cellular polarity in two different cell types, the epithelial cells (apicobasal polarity) and the oocyte (anteroposterior polarity) (Deng et al, 2003). Dystroglycan is also required non-cell-autonomously to organize the planar polarity of basal actin in follicle cells, possibly by organizing the laminin ECM. Taken together, these data suggest that the primary function of dystroglycan in oogenesis is to organize cellular polarity (Deng et al, 2003). Dystroglycan was previously demonstrated to be a major laminin cell

adhesion molecule in Schwannoma cells (Matsumura et al, 1997) and certain primary mammalian endothelial cell cultures (Shimizu et al, 1999). More recently, Peng *et al* published evidences showing that dystroglycan is also an important cell adhesion receptor in primary rat astrocytes and might play an essential role in process extension (Peng et al, 2008).

Dystroglycan and the integrins are the major ECM receptors. The integrins comprise a large family of heterodimeric transmembrane receptors each composed of a noncovalently associated alpha and beta subunit. Integrin-mediated adhesion to the ECM can activate many signaling pathways including the Erk, PI 3-kinase, FAK, Src and small Rho GTPases pathways, thus regulating a number of cellular events such as cell migration, cell cycle progression, gene expression and cell survival (Martin *et al*, 2002). Integrins are an important group of molecules for regulating astrocyte behaviour within the central nervous system. The $\alpha 1\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ heterodimers were found by our laboratory to be expressed in type 1 rat astrocytes (Tawil *et al*, 1993). In this study, the $\alpha 1\beta 1$ heterodimer was shown to mediate adhesion to laminin and collagen, $\alpha 5\beta 1$ to fibronectin in an RGD-dependent manner and $\alpha 6\beta 1$ to laminin. $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins were found in focal contacts whereas the $\alpha 1\beta 1$ integrin was localized in adhesion structures called point contacts. Since these integrin heterodimers share the same β subunit, it was proposed that the α subunit is involved in regulation of integrin accumulation within the two different types of cell-substratum contacts (Tawil *et al*, 1993). Other types of integrins have been found to play a role in astrocyte adhesion and migration. The alphaV integrins on primary astrocytes from both rat and mouse express just two members, alphaVbeta5 and alphaVbeta8, and are functional receptor for vitronectin. The alphaVbeta5 integrin is largely responsible for astrocyte adhesion to vitronectin while the alphaVbeta8 plays the dominant role in promoting migration on this substrate (Milner et al, 1999).

Astrocytes are the major glial cell within the central nervous system (CNS) and have a number of important physiological properties related to CNS homeostasis. They provide not only structural support and nutrition to the nervous tissues, but also affect neural function by the release of neurotrophic factors, contribute to the metabolism of neurotransmitters and regulate extracellular pH and K+ ion levels (Fields and Steven-Graham, 2002).

Injury to the CNS results in a characteristic astroglial scar which, in its final form, consists mainly of a meshwork of astrocytes tightly interwoven, and bound together by tight and gap junctions. The glial scar is essential to restore homeostasis of the CNS, but is inhibitory to nerve regeneration, acting as a physical barrier to axonal growth, and secreting extracellular matrix (ECM) molecules, such as chondroitin sulphate proteoglycans, that trigger an inhibitory growth response in neurons. The inflammation and the consequences of the wound healing process caused by the glial cell response to injury is thought to contribute to the overall morbidity and permanent disability that result from brain injury (Fitch and Silver, 2007).

In the early stage of glial scar formation, the astrocytes in the vicinity of the lesion acquire their reactive phenotype and become hypertrophic, overexpress GFAP, get polarized and produce many fine processes oriented towards the wound. The integrins are thought to play a major role in this process, where it has been shown that the interactions of an arginine-glycine-aspartic acid peptide sequence (RDG) dependent integrin with the ECM, in astrocytes at the leading edge of a lesion *in vitro*, lead to the activation and polarized recruitment of the small Rho GTPase Cdc42. This in turn recruits and activates the cytoplasmic mPar6/PKCζ complex, which is not required for protrusion formation, but is necessary for the correct orientation of the protrusion as well as the orientation of the microtubule organizing center (MTOC) (Etienne-Manneville and Hall, 2001). While significant advances have been made in understanding the precise sequence of molecular

events leading to astrocyte polarization and process extension, these signaling pathways are not completely elucidated.

Extracellular matrix receptors are thought to play an important role in astrogliosis. In the present study, we hypothesize that two types of ECM receptors, dystroglycan and beta(1) integrins, are differentially targeted to specific plasma membrane domains, in a time-dependent fashion, where they initiate signaling. In order to test this hypothesis, we used biochemical techniques combined with immunofluorescence microscopy, in the controlled environment of cell culture, to investigate the cellular distribution of the two ECM receptors and characterize the cytoskeleton of reactive astrocytes. Our analysis reveals that DG and integrins distribute differentially depending on the cell spreading stage, and both receptors get recruited to the cell leading edge in scratch-induced migration, possibly to GM1-rich lipid rafts. These results suggest that indeed, the cell distribution of ECM receptors to the plasma membrane is tightly regulated in astrocyte adhesion and proliferation.

The differential regulation of the actin and microtubule cytoskeletons in astrocyte proliferation was also investigated, using immunocytochemistry and cytoskeletal perturbing agents, and discussed in response to some of the issues raised in the work of Etienne-Manneville and Hall, 2001. One of the goals of the project was to decipher the role of DG in Rho GTPases signaling using the *in vitro* assay of astrocyte migration. In this respect, optimization of a Cdc42/Rac activation assay, adapted to DG-null and beta(1) integrin-null embryonic stem (ES) cell-derived astrocytes, was iniated and still on-going at the moment this project was terminated. Overall, the motive of the research project was based on the idea that a deeper understanding of the cytoskeleton reorganization coordinated by the ECM receptors would help elucidating the complex regulation of the mammalian astrocyte behaviour. This could contribute significantly in the understanding of how to attenuate the negative effects of astroglial scar formation on neural regeneration and improve the quality of life of many patients.

Chapter 2 – Literature Review

Astrocytes, first described by Ramon Y Cajal in 1913, have long been considered as simple support cells in the central nervous system. Over the last 20 years, the research on astrocytes has brought to light dozens of new discoveries that changed the long lasting paradigms. The cellular and molecular neurosciences have profoundly renewed the perception of cellular communication in the nervous system and the new paradigm of the tripartite synapse has emerged, whose basis is that neural activity is bidirectionnaly modulated by neuron and astrocyte activities. The astrocyte cell is now considered as an essential player in assisting the neural transmision of information and regulating a whole host of crucial processes such as neurotransmitter turnover, cerebral water transport and scar formation in the CNS. Astrocytes connect their cytoplasms to form a very complex network and communicate through this syncitium with calcium waves and chemical messengers. CNS wound repair involves the formation of a dense astrocyte syncitium through the process of astrogliosis, which is inhibitory physically and chemically to neuron regeneration. This review presents the biology of astrogliosis by focusing first on the astrocyte main ECM receptors and their cellular effectors. These molecules coordinate transduction signals to regulate cell behavior through their effect on cytoskeletal organization. In the last section of the review, a broader view of the CNS response to injury is exposed and the *in vitro* research model used to address the mechanisms of astrogliosis *in vitro* is described.

2.1 The Dystrophin Glycoprotein Complex

Dystroglycan was originally isolated from skeletal muscle as a central component of the dystrophin glycoprotein complex (DGC) (Ervasti et al, 1990). The DGC is a group of tightly associated transmembrane and cytoskeletal proteins that forms a molecular bridge between the F-actin cytoskeleton and the extracellular matrix,

thus stabilizing the muscle fibre sarcolemma against the forces of muscle contraction. The DGC is formed by dystrophin, dystroglycan complex (α- and β-DG), sarcoglycans (α, β, γ, δ, ε, ζ), sarcospan, syntrophins (α-1, β-1 and β-2) dystrobrevins, rapsyn, caveolin-3 and nitric oxide (NO) synthase (Figure 13) (Ervasti et al, 1991; Imamura et al, 2000; Nishiyama et al, 2004). These proteins are found in different combinations depending on the muscle type. Dystrophin is primarily expressed in muscle, while utrophin, its autosomal homologue, is expressed in a wide variety of tissues (Imamura and Ozawa, 1998). In the first section of this literature review, the nature and dynamics of the DGC components will be briefly reviewed with a particular focus on the dystroglycan subcomplex.

2.1.1 The dystroglycan subcomplex

2.1.1.1 Dystroglycan genetics

The human dystroglycan gene (*dag1*) is encoded by a 5.5 kb transcript found on chromosome 3 (3p21). There are two exons, one of 285 base pairs and the other one of 2400 base pairs, that are separated by a large intron (Ibraghimov-Beskrovnaya et al, 1993). The spliced exons code for a single propeptide which is cleaved and modified by posttranslational processing to yield two non-covalently associated subunits, α- dystroglycan (N-terminal) and β-dystroglycan (C-terminal) (Ibraghimov-Beskrovnaya et al,1992). A sequence comparison between the human, rabbit and mouse dystroglycan gene showed that these sequences are well conserved among these species (Brancaccio et al, 1994; Yotsumoto et al, 1996). However, the genomic DNA organization of the *dag1* gene may vary. For example, the dog *dag1* gene consists of three exons, with the translation start codon located in the exon 2 (Leeb et al, 2000). There have been no reported cases of mutations occurring in the human dystroglycan gene, thus suggesting its essential role in the cell (Higginson and Winder, 2005). In fact, disruption of the *dag1* gene in mice results in embryonic lethality and dystroglycan appears essential for the formation of the basement membrane (Reichert's membrane) that separates the embryo from the maternal circulation (Williamson et al, 1997).

2.1.1.2 Dystroglycan biosynthesis

Transport of proteins to the cell surface is a complex process involving glycosylation, folding and sorting. The proteins are first synthesized and assembled in the endoplasmic reticulum (ER) and then transported to the Golgi complex for further processing and maturation. Upon arrival at the *trans* Golgi network, they are sorted and packaged into post-Golgi carriers that move plus-end directed through the cytoplasm along microtubules to finally fuse with the cell surface (Lippincott-Schwartz et al, 2000). Although the process has not yet been elucidated entirely, it is thought to be the case for dystroglycan, which is transcribed as a single mRNA of approximatively 5,8 kb and translated into a 895-amino acid polypeptide: α-DG=1-653 and β-DG= 654-895 (Ibraghimov-Beskrovnaya et al, 1992; Bozzi et al, 2001; Bozic et al, 2004). Different glycosylated forms of dystroglycan can be detected in foetal and adult cardiac muscle, brain, kidney, liver and lung and in adult diaphragm, placenta, pancreas, skeletal muscle, stomach and more. Expression is most abundant in muscle and heart (Ibraghimov-Beskrovnaya et al, 1993).

A still unidentified mechanism cleaves the dystroglycan polypeptide into α-DG and β-DG, and the glycosylated proteins are then targeted separately to the plasma membrane (Holt et al, 2000; Esapa et al, 2003). The cleavage occurs between residues 653 and 654 within a SEA (Sperm, Enterokinase, Agrin)-homology domain with traits matching those ascribed to autoproteolysis (Akhavan et al, 2008). An intramolecular disulfide bridge between Cys669 and Cys713 and the resultant loop is critical for cleavage. The C-terminal region of α-DG (550-645) is also important for the cleavage (Deyst et al, 1995; Watanabe et al, 2007). The proteolysis and post-translational modifications of dystroglycan is essential, since it determines its functional state, and defects in these modifications are linked to muscular dystrophies and cancers in mice (Jayasinha et al, 2003; Akhavan et al, 2008).

2.1.1.3 Alpha-Dystroglycan

Electron microscopy analysis has revealed that α-DG has a dumbbell-like shape in which two globes (N-terminal and the C-terminal domain) are connected by an elongated rod corresponding to the central mucin-like region (Brancaccio et al, 1997). This mucin-like domain contains a large number of Ser/Thr residues and is extensively O-glycosylated (Brancaccio et al, 1998). In addition, α-DG has several conserved sites for asparagine-linked (N-linked) glycosylation and glycosaminoglycan addition (Durbeej et al, 1998). Among the glycans found on α-DG, we find O-linked mannose chains, including NeuAcα2, 3Galβ1, 4GlcNAcβ1 and 2Manα-O (Martin, 2003).

Circular dichroic spectral analysis showed the absence of typical secondary structure, i.e. alpha helix or beta sheet, and it was concluded that the N-terminal region of α-DG is an autonomous folding globular domain (Brancaccio et al, 1997). From the deduced amino acid sequence, the α-DG molecular mass is 74 kDa but the protein is highly glycosylated and depending on the tissue and developmental stage, its molecular mass varies from 120 to 180 kDa (Ibraghimov-Beskrovnaya et al, 1992).

α-DG interacts with several ECM molecules containing laminin G-like (LG) domains such as laminin, agrin, neurexin and perlecan. High affinity interactions with these molecules rely on these carbohydrate side chains in a calcium-dependent manner. In fact, full chemical deglycosylation of dystroglycan results in the complete loss of ligand binding activity (Ervasti et al, 1993). In 1997, a side chain that may be unique to α-DG was identified to mediate laminin binding: Sialic acid α2-3Galactose β1-4-N-acetylglucosamine β1-2Mannose-Serine/Threonine (Chiba et al, 1997).

Mutations in the dystroglycan gene itself do not lead to disease since they are thought to be embryonic lethal. On the other hand, mutations in genes involved in the glycosylation of α-DG, such as Fukutin, Fukutin-related protein (FKRP), O-

mannosyltransferase-1 (POMT1), O-mannosyl-β1,2-N acetylglucosminyltranserase-1 (POMGnT1), and glycosyltransferase (LARGE), result in muscular dystrophies (Muntoni et al, 2002; Beltran-Valero et al., 2002; Montanaro and Carbonetto, 2003; van Reeuwijk et al, 2007).

2.1.1.4 Beta-Dystroglycan

β-DG is a 43 kDa single transmembrane protein that binds non-covalently to α-DG through its N-terminal extracellular region (residues 654-750), whose structure is thought to be quite flexible and only partially folded (Di Stasio et al, 1999). The interaction with α-DG is independent of glycosylation (Sciandra et al, 2001). Within amino acid 880 to 895 of the C-terminal tail of β-DG, a PPxY motif can interact with the C-terminal WW-binding motif of dystrophin (Jung et al, 1995). Tyrosine phosphorylation in this PPxY motif appears to be essential in regulating the β-DG and dystrophin interaction (Ilsley et al, 2001; Pereboev et al, 2001) and this phosphorylation can be induced by agrin and laminin (Sotgia et al, 2003). It has been shown that β-DG also interacts with utrophin through its PPxY motif and this interaction is regulated by adhesion-dependent tyrosine phosphorylation (James et al, 2000). The muscle specific structural protein caveolin-3 also contains a WW-like domain that interacts with the PPxY motif of β-DG and can competitively block the interaction of dystrophin with β-DG (Sotgia et al, 2000). Structural proteins help maintaining the integrity of the cellular structure they support.

Phosphorylation at the PPxY motif seems to be a key mechanism for the regulation of β-DG interaction with its binding partners. Several studies have shown that tyrosine phosphorylation of the PPxY motif by Src-kinase blocks the interaction of β-DG with the WW domain of dystrophin and utrophin (James et al, 2000; Ilsley et al, 2001). This phosphorylation could act as a regulatory switch to inhibit the binding of certain WW domain containing proteins, while recruiting SH2 domain containing proteins (Sotgia et al, 2001). At least five SH2 domain containing proteins that interact with β-DG in a phosphorylation-dependent

manner have been identified: c-Src, Fyn, Csk, Nck and Shc (Sotgia et al, 2001; Sotgia et al, 2003). Another adaptor protein, the SH3 (Src homology 3) domain containing protein Grb2, involved in signal transduction and cytoskeleton organization, has been shown to bind β-DG (Yang et al, 1995) and this interaction can be inhibited by dystrophin in a range of 160 to 400 nM (Russo et al, 2000).

Tyrosine phosphorylation of β-DG also leads to profound changes in its sub-cellular localization. Upon phosphorylation, β-DG relocalizes from the plasma membrane to an internal membrane compartment, possibly a subset of recycling endosomes, where it colocalizes with c-Src, (Sotgia et al, 2003). In agreement with these findings, the GTPase Dynamin 1 was shown to interact with β-DG complexed with Grb2 in the CNS (Zhan et al, 2005). Dynamin 1 is essential in regulating endocytosis during synaptic vesicle release as well as constitutive endocytosis, which occurs in all cell types. This mechanism could be used by the cell to control dystroglycan levels at the cell surface.

Rapsyn is another DGC component that binds directly to β-DG. It is a 43 kDa cytoplasmic membrane-associated protein that tightly associates with the intracellular domain of acetylcholine receptors (AChR) through its coiled-coil domain. When rapsyn is knocked out in mice, the neuromuscular junction is disrupted and the mice die at birth because of a failure of neuromuscular transmission (Apel et al, 1997). The RING-H2 domain of rapsyn was shown to interact directly with the β-DG cytoplasmic domain. In non-muscle cells, rapsyn can also cluster β-DG. Thus, rapsyn may function as a direct link between nicotinic AChRs and the dystrophin/utrophin-associated glycoprotein complex that extends from the extracellular matrix to the cytoskeleton (Bartoli et al, 2001).

2.1.1.5 Beta-Dystroglycan in signal transduction complexes

Several studies have implicated directly β-DG in cell signaling. The unstructured proline-rich cytoplasmic tail of β-DG provides multiple sites for interaction with

signaling molecules. For example, ß-DG has been localized to microvilli structures in a number of cell types where it associates with the cytoskeletal adaptor ezrin, through which it is able to modulate the actin cytoskeleton and induce peripheral filopodia and microvilli via the Cdc42 pathway (Spence et al, 2004a; Batchelor et al, 2007). Interestingly, it has been shown that β-DG can also interact directly with F-actin through its cytoplasmic tail (Chen et al, 2003). In this study, DG overexpression in fibroblasts leads to a marked alteration of cell morphology, with the induction of numerous actin-rich surface protrusions around the cell periphery and on the apical surface, and a dramatic reorganization of the actin cytoskeleton.

In bovine brain synaptosomal extracts, a putative complex composed of β-DG, Grb2 and focal adhesion kinase p125^{FAK} FAK was suggested (Cavaldesi et al, 1999). FAK is a nonreceptor tyrosine kinase and a major mediator of integrin signaling pathways. Both FAK and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) phosphorylation are involved in the induction of the long-lasting increase in synaptic strenght called long term potentiation (LTP) (Yang et al, 2003). In neurons, FAK is not restricted to focal adhesion but distributed throughout the cell. These observations suggest the possible biological function of Grb2-β-DG interaction at the synapses where the adaptor protein Grb2 may mediate FAK-βDG interaction (Yang et al, 1995; Cavaldesi et al, 1999).

Another example of β-DG modulating signal transduction is its association with ERK and its upstream MAPK kinase (MEK) in fibroblasts. The mitogen-activated protein kinase (MAPK) cascade is a key intracellular signaling pathway that regulates diverse cellular functions including cell proliferation, cell cycle regulation, cell survival, angiogenesis, and cell migration. DG seems to regulate the adhesion-dependent activation of components of the Ras-Raf MAP kinase cascade by differentially targeting MEK to membrane ruffles and ERK to focal adhesions (Spence et al, 2004b). Ras activation is the first step in activation of

the MAPK cascade. As an effector of Ras, the Raf kinase is a component of signal transduction cascades of a variety of different stimuli such as growth factors, cytokines and hormones. At the cellular membrane, Raf proteins are attracted to GTP loaded Ras GTPases which might trigger profound structural changes similar to those found in Cdc42-associated Pak1 kinases (Kerkhoff and Rapp, 2001; Friday and Adjei, 2008).

The association of dystroglycan with components of the Ras-Raf MAP kinase cascade, including Grb2, MEK and ERK, and the tyrosine phosphorylation of dystroglycan in response to cell adhesion with recruitment of SH2 domain-containing adaptor proteins such as Shc, Nck and kinases of the Src family including Src, Fyn and FAK, suggest that DG has a key function in the transduction and modulation of various signaling cascades.

2.1.2 Dystrophin and utrophin

Dystrophin is a 427 kDa protein first identified in 1987 as the protein product of the Duchenne Muscular Dystrophy (DMD) locus since mutations in the X-linked dystrophin gene produce DMD (Hoffman et al, 1987). The dystrophin gene is one of the largest gene so far identified in humans, covering over 2,5 megabases and containing 79 exons. The gene contains 7 promotors that give rise to several tissue specific isoforms with a great variability in molecular weight. The corresponding 14-Kb dystrophin mRNA is expressed predominantly in skeletal, cardiac, smooth muscle and lower levels appear in the brain (Tinsley et al, 1994).

The WW and EF-hand domains of utrophin or dystrophin associate with the 20 terminal residues of the cytoplasmic tail of β-DG (Rosa et al, 1996; Rentschler et al, 1999). The WW domain is a small sequence of 38-40 semiconserved amino acids that is widely distributed among various structural, regulatory and signaling proteins (Sudol et al, 1995). The amino-terminal actin binding domain is responsible for anchoring dystrophin to cytoskeletal, filamentous actin (Rybakova et al, 2000). The extreme carboxyl-terminal region is α-helical in nature and

mediates its interaction with the syntrophins (Ahn et al, 1995) and can be phosphorylated by MAP kinase (Shemanko et al, 1995).

2.1.3 The Sarcoglycan subcomplex

The sarcoglycan subcomplex contains six sarcoglycans, α, β, δ, ε, γ, ζ, and copurifies as a complex within the DGC. Only ε-sarcoglycan is expressed in the brain where it accumulates on neurons as well as on astrocytes. All are transmembrane-spanning glycoproteins with at least one glycosylation site (Lapidos et al, 2004). Sarcoglycans are important for the stability of the DGC in general and the dystrophin-dystroglycan interaction in particular (Yoshida et al, 1999). The tyrosine phosphorylation of α- and γ-sarcoglycans is associated with muscle cell adhesion (Yoshida et al, 1998). The functional significance of these phosphorylation events is not known, but they suggest that sarcoglycans may also regulate cellular signaling pathways involving SH2 domain interactions (Rando, 2001). The absence of sarcoglycan leads to alterations of membrane permeability and apoptosis, two shared features of limb girdle muscular dystrophies (Hack et al, 2000). Due to an unknown mechanism of destabilization, mutations in a single sarcoglycan gene result in the loss of all sarcoglycans at the sarcolemma (Holt and Campbell, 1998).

2.1.4 Sarcospan

Sarcospan is a 25 kDa protein and a member of the tetraspanin family. It associates tightly with the DGC via the sarcoglycans and contributes to maintain the stability of the whole complex (Crosbie et al, 1999). Sarcospan is a highly hydrophobic protein that contains four transmembrane spanning helices with both N- and C-terminal domains located intracellularly (Crosbie et al, 1997). To date, only a single sarcospan gene has been identified. Studies of sarcospan protein expression have demonstrated that a major transcript is expressed primarily in skeletal, cardiac and smooth muscle, but a minor transcript is also expressed in other tissues such as ovary, prostate, and intestine. It seems to be absent from

neurons and nervous tissue (Imamura *et al.*, 2000). Sarcospan expression is dramatically reduced in muscle from patients with Duchenne muscular dystrophy.

2.1.5 Syntrophins

The syntrophins are a family of five ~60 kDA adaptor proteins and are encoded by separate genes. They are termed α, β1, β2, γ1, and γ2. Alpha syntrophin was shown to be the predominant isoform in skeletal muscle where it is localized on the sarcolemma and enriched at the neuromuscular junction. The beta syntrophins and γ2 syntrophin are also present in skeletal muscle but also are in most other tissues. The expression of γ1 syntrophin is mostly confined to brain (Alessi et al, 2006).

Syntrophin interacts directly with the carboxyl-terminus of both full-length and truncated forms of dystrophin. It is phosphorylated by a calcium–calmodulin-dependent protein kinase that regulates its binding to dystrophin (Madhavan et al, 1999). Each syntrophin isoform contains two pleckstrin homology (PH) domains: an N-terminal PH1 domain with an internal PDZ domain, and a PH2 domain. Syntrophin binds to PIP2, neuronal NO synthase (nNOS), calmodulin, and Grb2 (Oak et al, 2001). The different isoforms use their multiple protein interaction domains to localize a variety of signaling proteins to specific intracellular locations. It was shown that upon laminin binding, the DGC recruits Rac1 via syntrophin through a Grb2-Sos1 complex, thus identifying a putative signaling pathway for DGC in muscle homeostasis (Oak et al, 2003).

Syntrophins were also shown to interact with diacylglycerol kinase-zeta (DGK-zeta), which phosphorylates diacylglycerol to yield phosphatidic acid. Consistent with a role in actin organization, DGK-zeta and syntrophins were colocalized with filamentous (F)-actin and Rac in lamellipodia and ruffles. In adult muscle, DGK-zeta was colocalized with syntrophins on the sarcolemma and was concentrated at neuromuscular junctions (NMJs). Erk-dependent phosphorylation of DGK-zeta regulated its association with the cytoskeleton (Abramovici et al, 2003).

Syntrophin is a central organizer of the astrocyte dystrophin complex. It is required for proper localization of the water channel aquaporin-4 at the blood-brain barrier (BBB) and the enrichment of dystrophin in glial endfeet depends on the presence of α-syntrophin (Bragg et al, 2006). The water channel aquaporin-4 (AQP4) facilitates the rapid transfer of water across the BBB. Mice lacking α-syntrophin have reduced levels of AQP4 in perivascular astroglial endfeet resulting in reduced K+ clearance from the neuropil, leading to increased seizure susceptibility and reduced edema and infarct volume in brain trauma models (Puwarawuttipanit al, 2006).

2.1.6 Dystrobrevins

The dystrobrevins are a family of dystrophin-related proteins ranging in size from 59 to 78 kDa. There are at least two isoforms of dystrobrevin known in man, α-dystrobrevin and β-dystrobrevin (Rando, 2001). Alternatively spliced isoforms of both α- and β-dystrobrevin are expressed in brain, liver, and lung (Blake et al, 1998). Only α-dystrobrevin is highly expressed in skeletal muscle where it is localized along the sarcolemma and highly concentrated at the neuromuscular junction (Blake et al, 1996).

Dystrobrevins have a significant sequence homology with the cysteine-rich carboxyl-terminal region of dystrophin (Ambrose et al, 1997). The region of similarity with dystrophin includes several protein interaction domains, namely two EF hand-like domains, a ZZ domain, two syntrophin binding sites, and a pair of coiled-coil domains that mediate the interaction of dystrobrevin with dystrophin and utrophin. (Sadoulet-Puccio et al, 1997). It was shown that both α and β-dystrobrevin are specific phosphorylation substrates for protein kinase A (PKA) and that protein phosphatase 2A (PP2A) is associated with dystrobrevins. These results suggest a new role for dystrobrevin as a scaffold protein that may play a role in different cellular processes involving PKA signaling (Ceccarini et al, 2007). Moreover, Yoshida et al. provided further evidence that dystrobrevin may serve a signal transduction role by linking the signaling protein neuronal nitric oxide

synthase (nNOS) to the sarcoglycan-sarcospan complex via α-syntrophin associated with dystrobrevin (Yoshida et al, 2000).

α-Dystrobrevin-deficient mice were found to exhibit a muscular dystrophy phenotype, and showed secondary loss of nNOS from the sarcolemma despite the presence of other DGC components, such as ß-dystroglycan, α- and ß-sarcoglycan, dystrophin, and syntrophin (Grady et al, 1999). nNOS, which synthetizes nitric oxide, is responsible for increasing cyclic GMP levels to reduce vasoconstriction of smooth muscle. It also regulates many biological processes in the nervous system, including neurotransmitter release, plasticity, and apoptosis (Godfrey and Schwarte, 2003). α-Dystrobrevin–null mice were unable to increase cyclic GMP levels on stimulation, suggesting that nNOS may be a downstream signaling mediator of α-dystrobrevin (Grady et al, 1999).

2.2 Dystroglycan and the Extracellular Matrix

The extracellular matrix (ECM) is a highly organized multimolecular structure, essential for life in higher organisms. Due to its diverse nature and composition, the ECM can serve many functions, such as providing support and anchorage for cells, segregating tissues from one another, and regulating inter/intracellular communication. In this part of the review, some considerations will be addressed on DG's ligands, agrin, perlecan, neurexin, laminin and two other major ECM molecules, fibronectin and collagen.

2.2.1 Agrin

Agrin is a large (~200 kDa) heparin sulphate proteoglycan whose best characterized role is in the development of the neuromuscular junction during embryogenesis. It can be found in lung, kidney, central and peripheral nervous system, retina, and the immune system and appears to have varied functions at these sites (Groffen et al, 1998; Koulen et al, 1999; Khan et al, 2001). Agrin is encoded by a single gene, but the occurrence of RNA alternative splicing at three

sites and two alternate transcriptional start sites produces a wide number of proteins. RNA alternative splicing is an essential, precisely regulated post-transcriptional process that occurs prior to mRNA translation, in which exons can either be retained in the mature message or targeted for removal in different combinations to create a diverse array of mRNAs from a single pre-mRNA (Lynch, 2007). The core protein of agrin contains several motifs common to other proteins: there are nine Kazal type protease inhibitor domains, three laminin G like domains, and four epidermal growth factor domains (Tsim et al, 1992; Burgess et al, 2000). The protein also contains several sites for N- and O-linked glycosylation within the N-terminal portion. However, it is the C-terminus of agrin that is responsible for most of its biological activity at neuromuscular synapses. There are at least three confirmed receptors for agrin: α-DG, the muscle specific tyrosine kinase (MuSK) and integrin alphaVbeta1 (Gee et al, 1994; Martin and Sanes, 1997; Burgess et al, 2002). Agrin and laminin compete for the same binding site on dystroglycan (Yamada et al, 1996).

Agrin is expressed by neurons innervating skeletal muscle although the muscle itself secretes an isoform of agrin whose function remains unclear (Uhm et al, 2001; Williams et al, 2007). At the neuromuscular junction, agrin and its receptor MuSK are required for the formation of the postsynaptic apparatus and the aggregation acetylcholine receptors (AChR), for efficient transmission of signals across the neuromuscular synapse (McMahan, 1990). DG appears not to be required directly for agrin-MuSK signaling, since neural agrin-induced phosphorylation of MuSK was unaffected by treatments of anti-αDG antibodies which block agrin-αDG binding (Jacobson et al, 1998).

Agrin has also been implicated in several functions in the brain. However, the mechanism by which agrin exerts its effects in neural tissues is largely unknown. Nonetheless, biochemical evidences have shown that agrin binds to the alpha3 subunit of the Na+/K+-ATPase (NKA) in CNS neurons. Agrin inhibition of alpha3NKA activity results in membrane depolarization and increased action

potential frequency in cortical neurons in culture and acute slice (Hilgenberg et al, 2006).

2.2.2 Perlecan

Perlecan is highly conserved across species and has been found in all basement membranes. It is a large multidomain proteoglycan that consists of a core protein of molecular weight 450 kDa to which three long chains (each approximately 70-100 kDa) of glycosaminoglycans are attached. The core protein consists of five distinct structural domains. The N-terminal domain I (aa ~1-195) contains attachment sites for heparan sulphate (HS) chains. Although HS chains are not required for correct folding and secretion of the protein, lack of HS or decreased sulfation can decrease perlecan ability to interact with matrix proteins. Domain II comprises four repeats homologous to the ligand-binding portion of the LDL receptor with six conserved cysteine residues and a pentapeptide, DGSDE, which mediates ligand binding by the LDL receptor. Domain III has homology to the domain IVa and IVb of laminin. Domain V, which has homology to the G domain of the long arm of laminin, is responsible for self-assembly and may be important for basement membrane formation in vivo (Knox and Whitelock, 2006).

Perlecan binds with different affinities to many macromolecules. These include cell-surface receptors such as β1 and β3 integrins (Hayashi et al, 1992) and α-dystroglycan (Peng et al, 1998); other ECM proteins such as nidogen, collagen IV, laminin, fibulin and fibronectin (Talts et al, 1999); signaling molecules such as FGF2, FGF7, platelet-derived growth factor B (PDGF-B) (Mongiat et al, 2000); and enzymes such as acetyl cholinesterase (Peng et al, 1999). Thus, perlecan is involved in various biological activities, such as cell growth and differentiation, modulation of growth factor activity, lipoprotein binding, matrix assembly and cell adhesion.

2.2.3 Laminin

Laminins represent the most abundant glycoproteins of the basement membrane and display a high variability in their spatial and temporal expression during development or in the adult. Beside their fundamental role in organizing the basement membrane network, laminins promote several cellular processes such as adhesion, growth, polarization, differentiation and gene expression (Colognato and Yurchenco, 2000). The distinct biological activities of laminins depend on both the isoform type and the repertoire of laminin receptors expressed.

Laminins are large (400 to 900 kDa), cross-shaped, heterotrimeric glycoproteins that provide an integral part of the structural scaffolding of basement membranes in almost every animal tissue. All laminins are composed of individual α, β, γ subunits encoded on several different genes (Iivanainen et al, 1995). The three chains contribute to the α-helical coiled-coil that forms the long arm of the cross, whereas the short arms are composed of one chain each. The N-terminal globular domains at the tips of the short arms mediate the calcium-dependent polymerization of laminins 1 and 2 into a polygonal network (Colognato et al, 1999; Tisi et al, 2000).

Combinations of chains can generate at least 15 different laminin isoforms, expressed in a tissue-specific manner, giving rise to a great potential for functional diversity/specificity (Aumailley et al, 2005). To date, five α, three β and three γ chains are known for human and mouse (Miner and Yurchenko, 2004). All chains are glycosylated, and a few chains have been shown to have glycosaminoglycan side chains. The C-terminus of the laminin alpha chain contains a tandem of five laminin G-like (LG) domains. Many proteins contain LG modules, such as agrin, merosin, sex steroid binding protein, neurexin and perlecan. Laminin LG modules contain between two and four cysteines, with one pair forming a disulfide bridge close to the C-terminus. Most LG modules are modified by N-glycosylation, due to the presence of one to three consensus acceptor sequences (Timpl et al, 2000).

Laminins can polymerize through short arm interactions, bind to other matrix macromolecules such as agrin and contribute to cell differentiation, maintenance of tissue phenotypes, cell shape and movement, through binding to ECM receptors, mainly the integrins and dystroglycan. There is evidence that α-DG coordinately binds to the Ca2+ ion of the LG domains of laminin via its negatively charged oligosaccharides. In the alpha1 chain, the binding site of α-DG is mediated by LG4-LG5 globular domains in the COOH-terminal extension of the LN α1 and α2 chains and is contained within the E3 proteolytic fragment of laminin (Gee et al, 1993; Tunggal et al, 2000). LG3 is known to interact with the integrins and LG4 can promote cell adhesion and migration in epithelial cells (Chartier et al, 2006).

It has been reported that laminin can cluster DG on the surface of embryonic muscle cells in the absence of tyrosine phosphorylation (Cohen et al, 1997). These clusters contain dystrophin but are not accompanied by an accumulation of AChRs as it is the case with agrin-induced clustering. The laminin-induced clustering involved a corresponding depletion of DG in regions surrounding the clusters, suggesting that a subset of the DG molecules are mobile and can be aggregated through laminin self-polymerization.

2.2.4 Fibronectin

Fibronectins (FN) are large dimeric plasma glycoprotein found only in vertebrates. FN is widely expressed by multiple cell types and is critically important in vertebrate development, as demonstrated by early embryonic lethality of mice with targeted inactivation of the FN gene (George et al., 1993). They are involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion.

Although FN molecules are the product of a single gene, the resulting protein can exist in multiple forms that arise from alternative splicing of a single pre-mRNA

that can generate as many as 20 variants in human FN. FN usually exists as a dimer composed of two nearly identical ~250 kDa subunits linked covalently near their C-termini by a pair of disulfide bonds. Each monomer consists of three types of repeating units (termed FN repeats): type I (FNI), type II (FNII) and type III (FNIII) (Pankov and Yamada, 2002).

FN exists in a globular, soluble form in the plasma. A tightly controlled process transforms plasma FN to a fibrillar form within the ECM. The plasma form is synthesized by hepatocytes, and the ECM form is made by fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells (Vakonakis and Campbell, 2007). They are secreted by cells in an unfolded, inactive form and binding to integrins unfolds the fibronectin molecules, allowing them to form dimers so that they can function properly. Fibronectins bind collagen and cell surface integrins, causing a reorganization of the cell's cytoskeleton and facilitating cell movement. (Smith et al, 2007).

2.2.5 Collagen

Collagen is the single most abundant protein in the animal kingdom and is the major insoluble fibrous protein in the extracellular matrix and in connective tissue. There are at least 16 types of collagen, but 80 – 90 percent of the collagen in the human body consists of types I, II, and III (Brodsky et al, 2005). Collagen has a characteristic three residue repeat pattern, (Gly-Xaa-Yaa)(n), in its primary structure, which results in a stable, right-handed triple-helical conformation with the glycine residues at the core of the helix (Okuyama et al, 2006). Its rodlike domain has the potential for various modes of self-association and the capacity to bind receptors, other proteins, and nucleic acids. Collagen is exocytosed in precursor form (procollagen), which is then cleaved by procollagen proteinases to allow extracellular assembly (Vakonakis and Campbell, 2007). After post-translational modification, secreted collagen helices spontaneously self-assemble to cross-linked microfibrils and eventually mm-long fibrils to create large-scale molecular structures. Collagens are present in the ECM as fibrillar proteins and

give structural support to resident cells and help tissues withstand stretching. Cell adhesion to collagens is mainly mediated by the integrins alpha1beta1 and alpha2beta1, both of which bind a range of collagens including types I, IV and VI (Pozzi and Zent, 2003).

2.3 Integrins

The integrins are a large family of heterodimeric transmembrane cellular receptors, which mediate the association between the ECM and cytoskeletal proteins. They can modulate and control different cell functions such as adhesion, shape, polarity, growth, differentiation, motility, angiogenesis and coagulation, through the activation of several signaling pathways, including the Erk, PI 3-kinase, FAK, Src and small Rho GTPases (Martin *et al*, 2002).

Integrins heterodimers shared a wide variety of soluble and surface-bound ligands, including, laminin, collagen, fibronectin, vitronectin, tenascin, fibrinogen, thrombospondin, osteopontin, fibrillin, etc. Each integrin is composed of a non-covalently associated pair of alpha and beta subunits with a long extracellular domain, a hydrophobic transmembrane domain and a relatively short cytoplasmic domain of about 40-70 amino acids (Luo and Springer, 2006). The exception is the beta(4) subunit which has a cytoplasmic domain of 1088 amino acids, one of the largest known cytoplasmic domains of any membrane protein (Lipscomb et al, 2005).

2.3.1 The integrin superfamily

Humans have 18 α and 8 β subunits which assembles into 24 distinct heterodimers., For instance, the beta-4 subunit forms a heterodimer with the alpha(6) subunit only (Wilhelmsen et al, 2006). On the other hand, the beta(1) subunit forms heterodimers with at least eleven different alpha subunits, and binds to several different ligands. The alpha chain contributes importantly to the ligand specificity. For example, at least ten integrins bind to fibronectin (integrins

α3β1, α5β1, α4β1, αvβ1, α8β1, α4β7, αvβ6, αvβ8, αvβ3 and αIIbβ3), seven bind to laminin (α1β1, α2β1, α3β1, α6β1, α7β1, α10β1 and α6β4) and four bind to collagen (α1β1, α2β1, α10β1 and α11β1) (Humphries et al, 2006).

In type-1 astrocytes, the alpha(1)beta(1) integrin distributes to small (90-200 nm) punctate deposits called point contacts, whether the cells are spreading on collagen, laminin or fibronectin. In contrast, the same heterodimer accumulates in larger (2-6 μm) focal adhesion contacts in fibroblasts for the same conditions (Tawil et al, 1993). The alpha subunit thus appears to regulate the distribution of beta(1) integrins at adhesion sites in a cell specific manner and independently of its ligand. Focal adhesion contain clustered integrins and cytoplasmic proteins, such as FAK, talin, vinculin, paxillin and zyxin, that contribute to integrin connection to the actin cytoskeleton and signal transduction (Webb et al, 2002).

2.3.2 Bidirectional Signaling

Integrins use bidirectional signaling to integrate the intracellular and extracellular environments. In outside-in signaling, ligand binding activates intracellular signaling pathways (Giancotti and Ruoslahti, 1999). In inside-out signaling, signals received by other receptors activate intracellular signaling pathways, triggering a conformational change of integrin cytoplasmic domains, and consequently making the extracellular domain competent for ligand binding (Shimaoka et al, 2002). The switch to the high affinity state is rapid, with a subsecond time frame, is reversible in less than a minute, and is triggered from within the cell (hence the term inside-out activation) in response to extracellular chemical (Constantin et al, 2000) and/or mechanical stress signals (Zwartz et al. 2004b). Outside the cell plasma membrane, the alpha and beta chains lie close together along a length of about 23nm, the final 5 nm N-termini of each chain forms a ligand-binding region for the ECM (Tagaki et al, 2002). The two subunit cytoplasmic domains undergo significant spatial separation upon inside-out activation induced by G-protein-coupled receptors, phorbol ester or upon outside-in signaling induced by ligand binding (Arnanout et al, 2005).

2.4 Rho GTPases

2.4.1 The small Rho GTPases family

Rho GTPases are essential intermediates in the signaling pathways initiated by the ECM receptors. Rho (Ras homologous) family of GTPases belong to the Ras superfamily of small guanosine triphosphatases (GTPases) and are ubiquitously expressed and highly conserved throughout eukaryotes. They are cytoplasmic proteins implicated in the regulation of several cellular functions such as cell polarity, cell movement, cell shape, cell-cell and cell-matrix interactions, endocytosis and exocytosis, gene expression and cell cycle progression (Wennerberg et al, 2005). To date, 20 genes encoding different members of the Rho family have been identified in the human genome, and it is assumed that each one acts as a molecular switch to control distinct and overlapping biochemical pathways (Hall, 2005).

2.4.2 Rho, Rac and Cdc42

Rho GTPases are key regulators of actin and microtubule reorganization and are major mediators of integrin signaling in response to extracellular stimuli. Three of these GTPases, Cdc42, Rac (3 isoforms: Rac1, Rac2, Rac3) and Rho (3 isoforms: RhoA, RhoB, RhoC), have been studied in details. Typically, RhoA promotes actin stress fiber formation, focal adhesion assembly and microtubule plus end stabilization; Rac1 promotes lamellipodium formation and membrane ruffling; and Cdc42 promotes actin microspikes and filopodium formation. (Bishop and Hall, 2000; Hall, 2005). Rho GTPases are thought to shuttle between the cytosol and specific membrane sites upon extracellular stimulation. In polarized motile cells, Cdc42 and Rac1 localize to the leading edge of the cells. Cdc42 is also found at the Golgi apparatus, where it is thought to regulate establishment of cell polarity during secretory and endocytic transport. Unlike Cdc42 and Rac1, RhoA localizes mostly to the cytosol (Nalban et al, 2004; Irazoqui et al, 2004).

2.4.3 Cycling between GDP and GTP

Small RhoGTPases exhibit high-affinity binding for GDP and GTP. Their activation is determined by cycling between the GDP- and GTP-bound states. This process is controlled by three major classes of molecules known as guanine exchange factor (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). GEFs induce the exchange of GDP for GTP, thus promoting the activation of the GTPase, while GAPs promote hydrolysis of GTP into GDP. GPIs form a complex with the GTPase sequestering these molecules in the cytoplasm and preventing their membrane association (Degani et al, 2002). All RhoGEFs contain a Dbl-homology (DH) domain which encodes the catalytic activity, and an adjacent pleckstrin domain. The PH is thought to mediate membrane localization through lipid binding (Zeng et al, 1999). Reflecting their involvement in such a diversity of cellular processes, RhoA, Rac1 and Cdc42 proteins are each regulated by a great diversity of GEFs and GAPs (Lamarche-Vane et al, 1998; Raftopoulou and Hall, 2004; Grande-Garcia et al, 2005).

2.4.4 Mechanism of action

The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular autoinhibitory interactions, to expose functional domains within the effector protein. Upon binding to the Rho GTPase, the effector will remain active until GTP hydrolysis takes place. A modification of the effector such as autophosphorylation, as is the case for p21-activated kinase (PAK), phosphorylation by a separate kinase or binding to a different activating protein) may maintain activity even after dissociation of the GTPase. Inactivation of the effector occurs through removal of the modification (dephosphorylation or removal of a bound activating protein), allowing the effector to re-enter its inactive conformation. For example, the Rac/Cdc42 complex, which targets PAK1 and Ser/Thr kinases, have an intramolecular regulatory domain that inhibits kinase activity. Upon GTPase binding, the inhibitory sequence is displaced, leaving the kinase domain free to bind to substrates (Bishop and Hall, 2000).

2.4.5 Effectors

Almost all Rho GTPase effectors have multiple domains of interaction such as PH and SH3 domain, and many of these effector proteins also contain coiled-coil regions (ROCK, citron-kinase, IQGAP, mDia) (Hall and Nobes, 2000). Effectors of Cdc42 include PAK, Wiskott–Aldrich syndrome protein (WASP)/neuronal WASP, IQGAP1, Par6 and myotonic-dystrophy kinase-related Cdc42-binding kinase (MRCK). Effectors of Rac1 include PAK, IQGAP1 and phospholipase C. Some of these proteins, such as PAK and IQGAP1, interact with both Cdc42 and Rac1. Rho effectors include Rho kinase (ROCK), the myosin-binding subunit of myosin phosphatase, protein kinase N, mDia, citron and rhotekin (Raftopoulou and Hall, 2004). Many of these effectors are involved in the dynamics and organization of both the actin and the microtubule cytoskeleton and they all cooperate to regulate cell adhesion, cell polarity and cell motility.

2.5 The Plasma Membrane and Lipid Rafts

ECM receptors are mobile elements intermingled in the plasma membrane. The heterogeneity of biological signaling events forms the basis for conceptualizing the plasma membrane of cells as an array of microdomains of signaling molecules. The lipid composition of the plasma membrane bilayer and other endomembranes of living eukaryotic cells is extremely complex, consisting of up to 500 different lipid species, classified according to their head-group and backbone structure. These include neutral glycerolipids, glycerophospholipids, ceramides, glycosphingolipids and sphingomyelins (Edidin, 2003). A major lipid component of the plasma membranes is cholesterol or its closely related analog ergosterol. Cholesterol, having a semirigid tail, has a larger affinity for saturated lipids, and this is usually assumed to be the driving force of lipid rafts formation in living cell membranes (Gómez et al, 2008).

Lipid rafts are high order mobile microdomains (>100 nm) of plasma membrane which can be identified by their lower detergent solubility. They are characterized

by a dynamic assembly of cholesterol, sphingolipids, proteins, including caveolins, flotillins, src-family kinases, and glycosylphosphatidylinositol (GPI)-linked proteins (Mayor and Rao, 2004). The ganglioside GM1 is a structural component of lipid rafts widely used as a lipid raft marker and is detected with the use of cholera toxin B subunit, a GM1 specific protein, which has long been the only lipid probe of lipid rafts (Blank et al, 2007). The rafts are organized into platforms, and are endowed with membrane protein sorting and signal transduction functions. Their size and stability may dynamically change under specific signals or stimulus, contributing to the diversification of cellular responses.

Rho GTPases are able to interact with membranes via a posttranslational C-terminal geranylgeranyl lipid modification. In addition, RhoA and Rac1 are also thought to be concentrated in lipid rafts where they play an important role in integrin-mediated cell adhesion. Indeed, GTP-Rac1 binds more effectively to membranes from adherent than from suspended fibroblasts, suggesting that integrins regulate Rac1 membrane binding sites at the cell surface (del Pozo et al, 2004). Consequently, in nonadherent cells, GTP-Rac1 remains in the cytoplasm bound to RhoGDI and thus is uncoupled from its downstream signaling effector Pak. In addition, GM1-rich lipid rafts get internalized in non-adherent cells and translocate back to the cell membrane surface upon adhesion, an endocytic recycling mechanism that appears to be regulated by FAK and that may account for a variety of effects of integrins in adhesion-dependent cells (del Pozo et al, 2004). In the case of dystroglycan, it was associated with cholesterol-rich microsomal extracts which did not contain GM1 (Shah et a, 2006). It is unclear if there is one case in which dystroglycan is effective in the same membrane signaling microdomains than integrins.

2.6 The Cytoskeleton

The cytoskeleton is an elaborate network of protein fibers with their accessory proteins, pervading the cytosol of every cell, that help maintain cell shape, produce locomotion, provide mechanical strength, aid in chromosome separation during mitosis, and facilitate the intracellular transport of organelles. ECM receptors participate in the constant reorganization of three distinct cytoskeletal elements: actin microfilaments, microtubules and intermediate filaments. The actin cytoskeleton is thought to provide protrusive and contractile forces, along with associated motor proteins and microtubules, to form a polarized network allowing organelle and protein movement throughout the cell. Intermediate filaments are generally considered the most rigid component, responsible for the maintenance of the overall cell shape. (Etienne-Manneville, 2004)

2.6.1 Actin microfilaments

Actin is the most abundant intracellular protein in a eukaryotic cell, amounting for 1 to 10 % of the total cellular protein weight in nonmuscle cells. A typical cytosolic concentration of actin in nonmuscle cells is 0.5 mM. Actin is a moderate-sized protein consisting of approximately 375 residues, and is encoded by a large, highly conserved gene family (Lodish et al, 1997).

Actin exists as a globular monomer called G-actin and as a filamentous polymer called F-actin, which is a linear chain of G-actin subunits. Each actin molecule contains a Mg^{2+} ion complexed with either ATP or ADP (Le Clainche and Carlier, 2008). The actin molecule has two lobes with a cleft in between, where ATP or ADP is bound. The plus end of G-actin is the end that is opposite of the cleft that holds the ATP molecule. The minus end is the opposite end. Given a threshold concentration of actin monomers and ionic force, G-actin reversibly polymerizes into a structurally polar F-actin protofilament, a reaction facilitated by the hydrolysis of ATP-actin. Elongation occurs by addition of actin molecules to form a long helical polymer. After a period of growth, an equilibrium phase is reached

in which depolymerization controls the length as new monomers are added. Two parallel F-actin strands twist around each other in a helical formation, giving rise to the microfilaments of the actin cytoskeleton. Microfilaments measure approximately 7 nm in diameter with a loop of the helix repeating every 37 nm (Reisler and Egelman, 2007). Different dynamic microfilament structures are physically linked to distinct types of cell matrix adhesion complexes. These microfilament structures include lamellipodia, filopodia, stress fibers and podosomes. The cytoskeletal protein composition of each of these structures varies with cell type.

2.6.1.1 Cortical actin

In most cells, a rich area of actin filaments lies in the cortex, a narrow zone just beneath the plasma membrane. Besides supporting the plasma membrane, it interacts with a number of different proteins associated with the membrane such as the dystrophin glycoprotein complex as well as proteins in signaling pathways such as integrins. The intracellular proteins that are recruited by integrins have both actin binding activity and actin nucleating activity, and the assembly of the cytoskeleton in response to integrin activation is a critical step in the process of adhesion (Reichl et al, 2008). In many cell types, including fibroblasts but not astrocytes, cortical actin is synthesized through the activation of the Arp2/3 complex by WASP family proteins, where it nucleates branched actin filaments at the leading edge (Magdalena et al, 2003). Cortactin, an actin filament-binding protein, is also enriched at the leading edge of lamellipodia where it colocalizes with actin and Arp2/3 (Le Clainche and Carlier, 2008).

2.6.1.2 Stress fibers

Stress fibres are contractile cytoskeletal structures composed of bundles of approximately 10-30 actin filaments and myosin associated with focal adhesions. These structures are distributed in three classes: ventral stress fibers that are located at the ventral cell surface and associated with focal adhesions at both ends, dorsal stress fibers that are associated with focal adhesions at one end,

and transverse arcs that are not associated with focal adhesions. These bundles are held together by the actin-crosslinking protein α-actinin, although other actin-crosslinking proteins, such as fascin, espin and filamin, have also been detected in these structures (Chen et al, 1999). Stress fibers are connected to the cell membrane by anchoring at focal adhesions from which forces are transmitted throughout the cell via the motor protein myosin, which mediates sliding of anti-parallel actin filaments during stress fibers contraction (Hotulainen et al, 2006).

2.6.1.3 Treadmilling

Treadmilling is characterized by a net polymerization at the plus end of the actin filament and a corresponding depolymerization at the minus end of the filament, while the average polymer length does not change (Waterman-Storer and Salmon, 1997). The net result is a section of filament seemingly "moving" across the cytosol. This steady-state phenomenon is observed in many cellular cytoskeletal filaments, especially in actin filaments and microtubules. This process controls the formation of the lamellipodia at the leading edge and contributes to cell motility. Many actin binding proteins have been identified to accelerate the treadmilling cycle. For example, cofilin binds to the side of ADP-actin filaments and induces minus-end depolymerization to increase the concentration of monomeric actin at steady state. Then, profilin enhances the exchange of ADP for ATP to recycle the G-actin monomers and the profilin-actin complex assembles the monomers exclusively at the plus end (Rodionov and Borisy, 1997). In the lamellipodium of migrating cells, the acting nucleating factor Arp2/3 complex, activated by the signaling proteins WASP is also involved in this process in order to maintain the treadmilling rate constant by balancing the effect of capping proteins (Le Clainche and Carlier, 2008).

2.6.2 The Microtubule cytoskeleton

2.6.2.1 Structure

Microtubules (MTs) are one of the major components of the cytoskeleton and they are essential for cell division, cell migration, vesicle transport and cell

polarization. MTs are hollow tubes composed of 13 protofilaments of globular alpha and beta tubulin dimers organized in a head-to-tail fashion, yielding a helical cylindrical structure (Watanabe et al, 2005). GTP must be bound to both alpha and beta subunits for a tubulin heterodimer to associate with other heterodimers to form a protofilament or microtubule. MTs have a diameter of about 24 nm and length varying from 200 nm to 25 μm, more than twice the width of an intermediate filament and three times the width of a microfilament (Desai and Mitchison, 1997). The alpha and beta tubulin subunits are encoded by highly conserved separate genes, and both of these 55,000-MW monomers are found in all eukaryotes. A third member, γ-tubulin, has been shown to be specifically localized at the microtubule organizing center (MTOC) and has been implicated in the nucleation of microtubules in vivo (Burns, 1995). The MTOC is the structure found in eukaryotic cells from which microtubules emerge.

The inherent polarity of the alpha/beta tubulin dimmer confers to microtubules, like actin microfilaments, a (+) and a (−) end, which differ in their rates of assembly. Typically, MTs nucleate from their minus ends, which localize predominantly at the MTOC, near the nucleus in the center of the cell, while their plus ends point towards the cell periphery near the plasma membrane. As the motor molecules kinesin and dynein utilize this polarity, by moving to the plus and minus ends of the MT, respectively, MTs provide a system for the directional flow of information (Gundersen and Cook, 1999).

2.6.2.2 Microtubule-associated proteins

Microtubules are unstable polymers, and the plus end alternate stochastically between growing and shrinking states, a particular behaviour known as dynamic instability (Mitchison and Kirschner, 1984). Several proteins interact directly with unpolymerized tubulin and/or microtubules to modulate their dynamic properties. The microtubule associated proteins (MAPs) are tissue and cell type specific. They perform many different functions including both stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-

linking microtubules and mediating the interactions of microtubules with other proteins in the cell (Al-Bassam et al, 2002). The numerous identified MAPs have been largely divided into two categories: Type I including MAP1 proteins and type II, including MAP2, MAP4 and tau proteins (Permana et al, 2005)

One group of MAPs, the plus end binding proteins (termed +TIPs), have been shown to specifically accumulate at the plus ends of growing MTs and link MT plus ends to kinetochores, endocytic vesicles, and to the leading edge of migrating cells. They play a critical role in sensing cortical capture sites and linking the MT plus ends to these special cortical regions, a process essential for the establishment of cell polarity and directional migration. These +TIPS include CLIP-170, EB1, APC, IQGAP1 and the MT motors dynein and dynactin (Watanabe et al, 2004). IQGAP1, an effector of activated Rac1 and Cdc42, captures MTs at the leading edge via its carboxyl terminus, by binding to the CLIP-170 amino terminus, thus providing docking sites for MTs near the cell cortex, since IQGAP1 can bind and crosslink actin filaments with its amino terminus (Fukata et al, 1997). This mechanism could reinforce cell polarization by establishing polarized MT arrays (Fukuta et al, 2002).

2.6.2.3 Stable Microtubules

Many cell types contain a subset of 'stable', oriented microtubules, often termed Glu MTs, which differ from dynamic microtubules in many aspects. This MT population is distinguishable by their enriched level of two reversible posttranslational modifications of the tubulin subunit: removal of a tyrosine residue from the COOH terminus of α-tubulin, called detyrosination, and the acetylation of Lys40 of α-tubulin (Infante et al, 2000). Both detyrosinated tubulin (known as Glu tubulin after its newly exposed COOH-terminal Glu residue) and acetylated tubulin have been shown to accumulate in the subset of stable MTs in cells (Gurlang and Gundersen, 1995). Dynamic MTs contain tyrosinated tubulin (Tyr MTs), and can be differenciated from stable Glu MTs with the use of specific antibodies. Half-lives of Tyr MTs range from 5 to 10 min, while Glu MTs can last

for hours (Webster et al, 1987). Aside from being longer lived, Glu MTs are relatively more resistant to dilution-induced depolymerization and to depolymerization induced by the MT disrupting agent nocodazole (Palazzo et al, 2001).

Increased levels of Glu tubulin and acetylated tubulin in stable MTs are mostly observed in cells undergoing polarized morphogenetic events such as the directed motility of fibroblasts, neurite outgrowth, formation of myotubes, and during the formation of polarized tissues in embryos (Baas and Black, 1990; Gundersen et al., 1994). Since MTs act as 'rails' for transport, stable MTs may contribute to polarization by serving as unique tracks for motors, membrane transport, and intermediate filaments. Moreover, they can help to establish targeted delivery of vesicles and macromolecules to cellular subdomains, such as lipid rafts at the cell leading edges.

The serum factor lysophosphatidic acid LPA and integrin ligands fibronectin and vitronectin are known to promote the formation of stable MTs in spreading cells. In both cases, the integration of the transduction signal from the cell surface requires the small GTPase Rho. Integrin-mediated activation of FAK and its recruitment at the leading edge to lipid rafts is required for MT stabilization and capping by the Rho-mDia signaling pathway. The forming mDia (mammalian homolog of Diaphanous), an effector of Rho, has been shown to be sufficient to generate and orient stables MTs selectively near the leading edge in mouse fibroblasts migrating into a wound (Palazzo et al, 2001).

Cdc42 and Rac1 also contribute to MT stabilization. Upon integrin or growth factor activation, the effector of Cdc42 and Rac1, Pak, phosphorylates stathmin at Ser16. Stathmin is a MT-destabilizing protein, and phosphorylation at Ser16 blocks this activity in vitro (Daub et al, 2001). Therefore, Cdc42 and Rac1 can potentially stabilize MTs through this inhibitory mechanism. APC (adenomatosis polyposis coli) also contributes to the formation of Glu MTs by binding to the plus

ends of microtubules and stabilizing the growing ends, an activity that is decreased by phosphorylation of GSK-3β upon the upstream action of Cdc42 (Wen et al, 2004).

2.6.3 Intermediate filaments

2.6.3.1 Structure and function

Intermediate filaments are approximately 10-nm in diameter, intermediate in size between actin microfilaments and microtubules. In humans, there are at least 67 genes that encode functional intermediate filament (IF) proteins, which makes this gene family one of the largest in the human genome (Parrya et al, 2007). All cytoplasmic IF proteins have a common secondary structure, which consists of a central α-helical rod domain of about 310 amino acids that forms the core structure of the 10-nm filaments (Depianto and Coulombe, 2004). The rod domain is flanked by non-α-helical N- and C-termini that show great sequence variability, which translates in a broad range of potential interaction motifs. The intermediate protofilaments are produced by the dimerization of two rod domains to form a non-polar coiled coil. Cytoplasmic IFs dimers assemble into tetrameric filaments which then assemble into longer structure, forming a dynamic network that helps the cell withstand the mechanical stress. IFs are actively regulated by phosphorylation and other posttranslational modifications. IFs also interact with other cytoskeletal systems and act as scaffolds for various cell signaling molecules.

Intermediate filament proteins have been categorized into five distinct types based on their primary structure, gene structure, assembly properties and their developmentally regulated tissue specific expression patterns (Pallari and Eriksson, 2006). Type I to IV IF proteins, including keratin, vimentin, glial fibrillary acidic protein and neurofilament, form cytoplasmic IFs, whereas type V IF proteins, the lamins, constitute a filamentous network inside the nuclear membrane (Kim and Coulombe, 2007). Eventhough all intermediate filaments are

highly dynamic, none of them undergo treadmilling like microtubules and actin fibers.

2.6.3.2 GFAP

Astrocyte precursors usually express vimentin but glial fibrillary acidic protein (GFAP) becomes the major IF in mature astrocytes. It is therefore considered as a specific biological marker of these cells. GFAP is a class III IF protein that maps, in humans, to 17q21 (Ekny et al, 2007). GFAP can polymerize with itself or other type III or neurofilament proteins to form homodimers or heterodimers. Modulators of GFAP expression include several hormones such as thyroid hormone, glucocorticoids and several growth factors such as FGF, CNTF and TGF beta, among others (Buffo et al, 2008). GFAP is involved in many cellular processes, such as cell structure and movement, cell communication, astrocyte-neuron interactions and the functioning of the blood brain barrier (BBB) (Chang and Goldman, 2004). Indeed, disruption of the BBB results in the activation of native microglia with the recruitment of systemic inflammatory mononuclear cells to the lesion area (Lee et al, 2008). Upregulation of GFAP expression is one of the main characteristics of the astrocytic reaction commonly observed after CNS lesion.

2.7 Regulation of Cell Behaviour

Cell adhesion, polarization and migration are fundamental processes involved in wound healing. The Rho family GTPases, particularly Rho, Rac and Cdc42, play pivotal roles in regulating cell behaviour by controling the signal transduction pathways activated by the ECM receptors to promote cytoskeleton reorganization.

2.7.1 Cell adhesion

Adhesion to the ECM is a critical step for cell spreading and regulation of cell motility. Cellular adhesion is the binding of a cell to another cell or to a surface or matrix. Studies have shown that cells do not attach uniformly to a surface but rather at specialized foci, of which the largest have been termed focal contacts, or focal adhesions. The classification of adhesion structures depends on their size, shape, intracellular localization, molecular composition, and dynamics. These cellular adhesions can be classified into point contacts, focal complexes, focal adhesions, and fibrillar adhesions. Adhesion turnover is regulated by specific cell adhesion molecules that interact with the complex molecular assemblies connected to the cytoskeleton as previously described.

2.7.1.1 Point contacts

Point contacts are smaller (90-200 nm) and more punctate in appearance than fibroblastic focal adhesions. In neuron, point contacts are integrin-dependent adhesion sites which contain many of the same proteins that localize to focal adhesions in non-neuronal cells, including paxillin, vinculin, β1-integrin, and FAK (Renaudin et al, 1999). The Rho GTPases RhoA and RhoB are enriched at neuron point contacts and the coordinated activities of Rho, mDia, ROCK, Rac1 and FAK support rapid neurite outgrowth through stabilization of lamellar protrusions (Woo and Gomez, 2006).

In rat astrocytes, the early stages of cell attachment and spreading are mediated by integrins, including alpha(1)beta(1), which is found constitutively in point contacts. Astrocyte point contacts are closely apposed (15 nm) to the substratum and contain clathrin but rarely codistribute with actin or vinculin (Tawil et al, 1993; Nermut *et al*, 1991).

2.7.1.2 Focal adhesion

Focal adhesion complexes ensure that the ECM is firmly connected to the actin cytoskeleton. Focal adhesions are bigger (2-5 μm), more stable and display a

slower turnover than point contacts and focal complexes. They are located at the cell periphery and more centrally in less motile regions, associated with the end of stress fibers and sometimes acting as mechano-sensors. These structures contain high levels of vinculin, talin, paxillin, zyxin, α-actinin, VASP, Src, FAK, phosphotyrosine proteins, and integrin heterodimers (Webb et al, 2002).

FAK is a cytoplasmic non receptor tyrosine kinase and colocalize with integrins in focal adhesions in adherent cells. FAK appears also to regulate GM1 localization to the cell surface (Palazzo et al, 2004). Its activation and tyrosine phosphorylation is dependent on the binding of integrins to their extracellular ligands in a variety of cell types. In migrating fibroblasts, focal complexes first form at the leading edge of lamellipodia and filopodia induced by Rac1 activity. Subsequently, in a process that requires RhoA activity and actomyosin contraction, focal complexes mature into larger focal adhesions as additional proteins are recruited, linking the adhesion site to the actin cytoskeleton (Nobes and Hall, 1995). Although the assembly/disassembly processes are relatively fast, the redistribution of focal adhesions is determined by diffusion of free integrins and occurs on the slow time scale of one hour range (Novak et al, 2004).

2.7.2 Cell Polarity

The process of cell polarization involves the formation of polarized MT arrays through the production of stable microtubules, and the translocation of the MTOC (or centrosome) and the Golgi to a position between the nucleus and the free edge of the cell. Cdc42 has been shown to regulate MTOC reorientation in migrating fibroblasts, astrocytes and endothelial cells (Etienne-Manneville and Hall, 2001; Cau and Hall, 2005). The signaling pathways and the cytoskeletal dynamic regulating cell polarity and protrusion formation are distinct and may differ from one cell type to another.

2.7.2.1 Polarity in astrocytes

In the migrating astrocyte, MTOC polarization and protrusion formation is mainly driven by the microtubule network. In addition, Golgi polarization is actin independent in these cells and doesn't require neither Arp2/3 nor any WASP-family proteins (Magdalena et al, 2003). This is different from NIH 3T3 mouse fibloblasts for which polarization of Golgi involves the local activation of the Arp2/3 complex, an actin nucleator, at the cell membrane by Wasp/Wave family members which are themselves major targets for Cdc42 and Rac, underlining that polarity is likely to have cell type-dependent mechanisms (Etienne-Manneville and Hall, 2001).

The classical *in vitro* model to study astrocyte polarity consist of making a scratch with a blunted needle through an astrocyte monolayer to simulate a lesion. In astrocytes, centrosome position seems to be a good indicator for the orientation of the protrusion and therefore it can be used to investigate the pathways involved in cell polarization. After "wounding", the cells at both sides of the scratch re-orient the MTOC and Golgi apparatus and extend processes to close the empty space. The movement of the MTOC and the Golgi can be visualized using their respective immunofluorescent probes which targets the MTOC marker pericentrin and the Golgi-associated coatomer protein beta-COP.

In vitro stimulation of polarization in astrocytes with either the serum factor lysophosphatidic acid (LPA) or a scratch assay (see section 2.10), results in the recruitment and activation of Cdc42, at the leading edge of the reactive cells, through the action of integrins. Cdc42 in turn activates its target complex, mPar6/PKCz, which is not required for protrusion but is required for the correct orientation of the protrusion as well as the MTOC (Etienne-Manneville and Hall (2002; 2003). Interestingly, and in contrast to other cell types, this relocalization during migration does not involve Par3. Using FRET analysis, it has been shown that aPKCz activation within the Par6 complex is mediated by Cdc42-GTP through a double-negative regulation, whereby activated Cdc42 inhibits the Par6-

dependent inhibition of aPKCz (atypical protein kinase C zeta). Par6 constitutively inhibits aPKCz within the complex until Cdc42 releases the inhibition. The strategy is thus to target the inactive Par6-PKCz complex to a defined cortical domain and regulate its timely activation by integrin-mediated activation of Cdc42 (Henrique, 2003).

The proteins Scrib and the GEF betaPIX control Cdc42 activation and localization during astrocyte polarization (Osmani et al, 2006). Cdc42 triggers microtubule reorganization through GSK-3β (glycogen synthase kinase 3β) and APC (adenomatous polyposis coli), two components of the Wnt pathway. GSK-3 is constitutively associated with the complex Par6-PKCz. Cdc42 activates this complex, leading to the inactivation and subsequent dissociation of GSK-3β by phosphorylation (Etienne-Manneville and Hall, 2003). This restricted spatial inactivation of GSK-3β may allow APC to binds to the plus ends of microtubules and stabilizes the growing ends. +TIPs, including CLIP-170 and APC, are thought to function as capturing devices at specialized cortical regions. APC can associate with microtubules directly through residues located in the carboxy-terminal region or it can bind MTs through EB1 (Etienne-Manneville et al, 2005). Activation of the Par6–PKCz complex by Cdc42 at the leading edge of migrating cells promotes both the localized association of APC with microtubule plus ends and the assembly of Dlg-containing puncta at the plasma membrane.

hDlg1 is the human orthologue of Drosophila disc large protein. This 100 kDa protein binds the carboxy-terminal end of APC via its PDZ domain (Matsumine et al, 1996; McLaughlin et al, 2002). The physical interaction between APC and Dlg1 is required for polarization of the microtubule cytoskeleton (Etienne-Manneville et al, 2005).

IQGAP1, an effector of Rac1 and Cdc42, interacts with CLIP-170 and binds to APC. IQGAP1 can also link APC to actin filaments in vitro. Activation of Cdc42, in response to the polarizing signal, leads to the formation of the complex IQGAP1-

APC-CLIP-170, that links the actin cytoskeleton and microtubule dynamics during cell polarization and migration (Watanabe et al, 2004). The local traction forces, generated on the microtubule array by the microtubule-based motor protein dynein, could provide the basic mechanism through which the MTOC aligns in front of the nucleus (Palazzo et al, 2001). The reorientation of the centrosome or MTOC towards the direction of migration facilitates cell migration by directing transport pathways to the leading edge and is particularly important to achieve efficient and persistent migration over long distances (Raftopoulou and Hall, 2004).

2.7.3 Cell Protrusion formation and Migration

Cell migration is a highly orchestrated multistep process essential in all multicellular organisms. It is important not only during development, but also throughout life such as in wound repair, angiogenesis, immune surveillance and invasive pathologies. In the cycle of migration, a cell first acquires a characteristic polarized morphology by reorienting the MTOC and the Golgi in response to chemotactic or other migration-promoting agents. This rotation of the Golgi provides to the newly formed leading edge a vectorial flow of Golgi-derived vesicles. At the cell front, actin and microtubule assembly drives the extension of lamellipodia and filopodia. At the leading edge of the lamellipodium, the cell forms focal adhesions that connect the extracellular matrix to the actin cytoskeleton to provide the traction force for migration. Focal adhesion formation at the front of the cell and disassembly at the rear are critical for the migration of many adherent cells. The traction forces at focal adhesions result from actomyosin contraction and are in the range of tens of nano-Newtons (Wu et al, 2005). Finally, to move forward, the cell retracts its trailing edge by combining actomyosin contractility and disassembly of adhesions at the rear (Raftopoulou and Hall, 2004).

2.7.3.1 Protrusion formation and migration in astrocytes

Understanding astrocyte migration is fundamental to elucidate the mechanisms behind the formation of the glial scar. In migrating astrocytes, the formation of protrusions is a microtubule-driven process that involves polarization of the MTOC, microtubule extension at the leading edge, and intracellular fluid movement.

In the *in vitro* scratch wound model of astrogliosis, the recognition by integrins of the new empty space made by the blunted needle appears to be the first polarity signal which rapidly leads to the activation and localized recruitment of Cdc42. Cdc42 then establishes and maintains the polarity of the migrating cell by promoting Rac-dependent protrusions and aPKCz/ dynein-dependent reorientation of the MTOC, Golgi, and microtubule network toward the leading edge. Interestingly, Cdc42-mediated initiation of protrusion formation is independent of mPar6 and aPKCz. Nevertheless, inhibition of aPKCs with the GF109203X inhibitor leads to a disorganized orientation of protrusions (Etienne-Manneville and Hall, 2001). It seems likely that suppression of MTOC reorientation leads to mislocalized targeting of proteins required for protrusion formation.

The formation of protrusions, which lead to the elongated morphology, requires Cdc42 only at early times after wounding. Rac, on the other hand, is essential for both the development and the maintenance of protrusions during migration (Etienne-Manneville and Hall, 2001). Spatially restricted Rac activity is required at the front of the cell to regulate cortical actin polymerization and membrane protrusion. In opposition to Cdc42 and Rac activity, Rho signaling doesn't promote astrocyte protrusion formation. Using *in vitro* scratch-wound assays, it has been shown that inhibition of Rho by either direct inhibition with bacterial $C3^{bot}$ protein or by blocking downstream targets such as ROCK, leads to enhanced process formation, migratory activity and faster wound closure in astrocyte monolayers at any time points (Höltje et al, 2005).

Damage to brain cells causes the release of cytoplasmic nucleotides, such as ATP and uridine 5'-triphosphate (UTP), which are ligands for P2 nucleotide receptors. Studies with primary rat astrocytes indicate that activation of a G protein-coupled receptor for ATP and UTP increases GFAP expression and both chemotactic and chemokinetic cell migration (Wang et al, 2005). UTP in turn increases the expression of alpha(V)beta(3)/beta(5) integrins and vitronectin, an extracellular matrix protein that is a ligand for these same integrins. The $P2Y_2$ receptor ($P2Y_2R$), a subtype of P2 receptors, contain an arginine-glycine-aspartic acid (RGD) sequence that enables this receptor to interact selectively with alpha(V)beta(3) and alpha(V)beta(5) integrins to modulate its function (Erb et al, 2001). Moreover, anti-α_V integrin antibodies prevented UTP-stimulated astrocyte migration (Wang et al, 2005). $P2Y_2R$-mediated astrocyte migration requires the activation of the phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (Akt) and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathways (Bagchi et al, 2005). In fact, time course immunocytochemical studies showed that scratching human astrocytes induced the activation (phosphorylation) of ERK 1/2 at 10 min after scratching. It was concluded that the migration of human astrocytes after injury is partly initiated by activation of the MEK-ERK signaling pathway, in addition to the Rho GTPase pathway (Lim et al, 2007). These two pathways seem to coordinate together, since recent evidences showed that upon release of ATP, P2 receptors are coupled to GSK3beta by a PKC-dependent pathway (Neary et al, 2006)

Aquaporin-4 (AQP4) is the major water-selective channel in astroglia throughout the central nervous system and facilitates water movement into and out of the brain. In astrocytes, it colocalizes with dystroglycan in laminin-induced aggregation of AQP4 channels (Guadagno and Moukhles, 2004) and both proteins co-purified in a complex using sucrose density gradient ultracentrifugation (Nicchia et al, 2008). AQP4 was found to be upregulated in reactive astroglia and was polarized to the leading edge of the plasma membrane in the migrating astrocyte. AQP4 knockout or knockdown in astroglial cell cultures

was found to greatly impair their migration towards the site of injury in the *in vitro* scratch wound model. Interestingly, AQP4 deletion did not affect other astroglial functions, such as adhesion or proliferation. Loss of AQP4 probably reduced membrane water fluxes that occur normally during cell migration (Saadoun et al, 2005). Thus, AQP4 has a key role for astrocyte migration and modulation of AQP4 expression/function in the CNS could be considered as a novel strategy to control glial scar formation (Nicchia et al, 2008).

2.8 Biology of astrocytes

2.8.1 The glial cells

The two main classes of cells in the vertebrate nervous system are the neurons and glia. Glial cells are divided into two major classes: microglia and macroglia. Microglia are the smallest of the glial cells. The microglial cells are constantly moving and analyzing the CNS for damaged neurons, plaques, and infectious agents. Some act as phagocytes cleaning up CNS debris but most serve as representatives of the immune system in the brain (Kandel et al, 2000).

Macroglial cells can be divided into three types: oligodendrocytes, Schwann cells and astrocytes. Oligodendrocytes are found in the CNS while Schwann cells occur in the PNS. Both of them are responsible for insulating neuronal axons, forming a myelin sheath by tightly winding their membranous processes around the axon in a spiral. The myelin sheath is an electrically-insulating dielectric layer that surrounds only the axons of many neurons. It aids in the quick and accurate transmission of electrical current carrying data from one nerve cell to the next. Some evidence suggest that dystroglycan may play a role in this process. After blocking α-DG function, oligodendrocytes failed both to produce complex myelin membrane sheets and to initiate myelinating segments when co-cultured with dorsal root ganglion neurons (Colognato et al, 2007). In addition, the loss of dystroglycan from Schwann cells causes myelin instability and disorganization of the nodes of Ranvier. Thus, oligodendrocytes and Schwann cells express and

use dystroglycan receptors to regulate myelin formation. Although the types of myelin produced by oligodendrocytes and Schwann cells differ to some degree in the chemical makeup, myelin typically consists of 70% lipids (cholesterol and phospholipid) and 30% proteins (Simons et al, 2007).

2.8.2 Description and function of astrocytes

Astrocytes are the most numerous of glial cells and are roughly ten times more abundant than neurons, a ratio that has increased during evolution. They perform many functions, including biochemical support of endothelial cells which form the blood-brain barrier, the provision of nutrients to the nervous tissues, control of blood flow, and a principal role in the repair and scarring process in the brain. Astrocytes constitutively express multiple ion transporters and neurotransmitter receptors, and release soluble factors to promote neuronal signaling, indicating an important role in neuronal excitability. They are characterized by an irregular, star-like shape, with rather long processes, some of which terminate in broad end-feet that allow the astrocyte to be in contact with both capillaries and neurons, in order to provide neural cells structural, metabolic and trophic support, as well as regulating the extracellular pH and K+ ion levels (Lee et al, 2006; Volterra and Santello, 2008).

At the synapse, astrocytes and neurons interact to modulate the release of neurotransmitters in the synaptic cleft, thus integrating the information transmission between glia and neurons (Halassa et al, 2007). Since many of the astrocyte's numerous cellular protrusions envelope synapses made by neurons, astrocytes form a "tripartite" complex with presynaptic and postsynaptic structures and regulate synaptic transmission and plasticity by sensing neuronal activity and releasing neuroactive molecules. As non-excitable cells, astrocytes are unable to generate action potentials and to communicate via electrical signals. Nevertheless, astrocytes respond to a variety of neurotransmitters and modulators released during synaptic activity by activating the corresponding ligand-gated receptors followed by an increase or a decrease of their intracellular

Ca(2+) concentration ([Ca(2+)]), mostly via release of Ca2+ from the internal store. Astrocytes can release gliotransmitters, such as glutamate, in response to [Ca(2+)] elevations (Jourdain et al, 2007).

Astrocytes are linked in a network by gap junctions, creating an electrically coupled syncytium. An increase in intracellular calcium concentration can propagate outwards through this syncytium and consequently signal to adjacent neurons. Mechanisms of calcium wave propagation include diffusion of IP3 through gap junctions and release of extracellular signaling molecules, particularly adenosine triphosphate (ATP), with subsequent activation of purinergic membrane receptors on neighboring cells (Newman, 2001). Both glutamate and ATP can also be released by [Ca(2+)] –independent mechanisms. Astrocytes have extensive contact with the vasculature and therefore are well positioned to modulate vascular function. Ca2+ signaling in astrocytes has been reported to evoke both vasodilation and vasoconstriction in arterioles, effects that may involve distinct mechanisms of astrocyte activation and distinct extracellular messengers (Gordon et al, 2007). The significance of the different [Ca^{2+}] increase events generated in astrocytes by neuronal activity, starting from their amplitude, frequency and extent of propagation remains largely unclear.

2.8.3 Astrocytes and dystroglycan

In wild-type mice, dystroglycan has been described on the foot processes of astrocytes in the microvasculature and on pial surfaces, and in 'neuronal elements' of the hippocampus and cerebellar cortex (Michele *et al*, 2002). In the GFAP-Cre/DG-null mice (a conditional knockout of *α*- dystroglycan tied to an astrocyte-specific promoter), the loss of dystroglycan from astrocyte foot processes at the glia limitans leads to basal lamina abnormalities, underlined by a strong reduction in total laminin binding of the DG-null brain associated with discontinuities in the glia limitans and a type of glial hypertrophy (Moore et al, 2002). A reactive inflammatory gliosis is also observed in the cerebral cortex of

GFAP-Cre/DG-null mice, possibly caused by the disruption of the glia limitans between the brain parenchyma and the subarachnoid space (Moore et al, 2002).

Beside its role in cell adhesion, astrocyte DG seems to be responsible for the aggregation of the potassium channel Kir4.1 and the aquaporin AQP4 in response to laminin, thus contributing to the regulation of the extracellular volume of the brain and the permeability of the blood – brain barrier (Guadagno and Moukhles, 2004). Ligand-binding to the highly glycosylated isoform of α-DG in concert with α- and β1-syntrophins is crucial for the polarized distribution of Kir4.1 and AQP4 to functional domains in brain cells (Rurak et al, 2007).

2.8.4 Astrocytes and integrins

Integrins are highly expressed by glial cells both in the PNS and CNS. For instance, PNS Schwann cells express at least $\alpha1\beta1$, $\alpha6\beta1$, $\alpha v\beta8$, $\alpha2\beta1$, $\alpha6\beta4$, $\alpha5\beta1$, $\alpha1\beta3$ (Previtali et al, 2001). Astrocytes express a wide variety of integrin subunits, including $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$ αV and $\beta1$, $\beta3$, $\beta4$, $\beta5$, $\beta8$ chains and a few examples are presented here. Most of these results have been found using function blocking antibodies to the integrin subunit and RGD peptides.

As previously described, the $\alpha1\beta1$, $\alpha5\beta1$ and $\alpha6\beta1$ heterodimers were found to be expressed in type 1 rat astrocytes as they mediate cell adhesion to collagen fibronectin and laminin. Type 2 astrocytes do not express $\alpha1\beta1$ heterodimers (Tawil *et al*, 1993). Another integrin, $\alpha6\beta4$, has been suggested to participate in the big-h3-mediated astrocyte cell adhesion (Kima et al, 2003). big-h3 is a secretory protein that is induced by transforming growth factor (TGF)-b. Kima *et al* have shown that big-h3 expression is induced in cultured astrocytes by TGF-b1 and in rat cerebral cortex by stab wound . The protein big-h3 may transduce intracellular signals through the focal adhesion proteins, which may regulate certain aspects of astrocyte response to brain injury.

Vitronectin is another integrin ligand express by astrocytes. Vitronectin is an heteromeric glycoprotein (65 and 75 kD) involve in cell adhesion that binds the integrin via its RGD-sequence peptide. Milner *et al* have shown that both αvβ5 and αvβ8 can act as functional receptors for vitronectin. Furthermore, their data provide the first evidence that αvβ8 regulates migration and show that astrocyte αvβ5 and αvβ8 integrins have distinct functions (Milner et al, 1999).

In response to focal stroke, astrocytes in the peri-infarct region become activated and osteopontin, a provisional matrix protein induced during wound healing, is expressed by microphages and macrophages. By 15 days, astrocytes expressing integrin receptor αvβ3 are localized in an osteopontin-rich region concomitant with formation of the new glial-limiting membrane, suggesting a role for integrin αvb3 in astrocyte wound healing in vivo (Ellison et al, 1998). Further studies are needed to confirm the presence, in these osteopontin-rich regions, of $P2Y_2R$, a purinergic receptor known to modulate αvβ3.

2.9 CNS Injury and the Formation of the Glial Scar

2.9.1 Astrogliosis

In contrast to the PNS, central nerve fibers are unable to regenerate after injury. Regeneration in the CNS fails mostly because of the combined consequences of molecular inhibitors of axonal growth released in the lesion site and the unstable oxygen and nitrogen species commonly generated during the respiratory burst of the innate immune response, which render neurons particularly susceptible to cell death.

CNS damage and inflammation triggers an evolutionary conserved glial reaction, or astrogliosis, that initiates a serie of cellular and molecular events that evolve over several days and lead eventually to the formation of a typical glial scar. The glial reaction to injury is broadly the same whatever the source of injury and

results in the recruitment to the lesion site of macrophages from the blood stream, astrocytes, microglia, oligodendrocyte precursors and in some cases, involvement of meningeal cells and stem cells. Astrocytes acquire a reactive phenotype characterized by increased expression of the structural filament proteins, GFAP and vimentin, and polarization and migration towards the lesion site. The resulting glial scar is an evolving structure acting as a physical and molecular barrier to regeneration at lesion sites. The final structure is predominantly astrocytic, along with ECM molecules such as the inhibitory extracellular matrix molecules known as chondroitin sulphate proteoglycans (CSPGs). These cells divide and interdigitate their processes to create dense plexus – a process called anisomorphic gliosis (Laird et al, 2008) – eventually filling the vacant space, which is often accompanied by a fluid-filled cyst (Pekny and Nilsson, 2005).

2.9.2 Inhibitory factors

CSPGs are a family of molecules characterized by a protein core to which are attached large, highly sulphated glycosaminoglycan (GAG) chains. After injury, CSPGs (aggrecan, brevican, neurocan, versican, phosphacan and NG2) and laminin expression is rapidly upregulated by reactive astrocytes, forming an inhibitory gradient that is highest at the centre of the lesion and diminishes gradually (Fawcett et al, 1999).

The myelin structure formed by oligodendrocytes that normally ensheaths nerve fibres can become damaged after injury, exposing severed axons to myelin-associated inhibitors realeased from intact oligodendrocytes and myelin debris which can restrict axon regrowth. Indeed, CNS myelin was found to be inhibitory to axon outgrowth as well as several other molecules like myelin-asociated glycoprotein (MAG), oligodendrocyte myelin glycoprotein, the transmembrane semaphorin 4D and ephrin B3 (Yiu and He, 2006).

Another myelin associated protein that contributes to neurite outgrowth inhibition is the Nogo protein (Fournier et al, 2001). Nogo expression is increased by oligodendrocytes at the borders of CNS lesion sites (Hunt et al, 2002). It binds with high affinity to its GPI-linked axonal surface receptor Nogo-66 (NgR), which contains a 66-residue extracellular domain. The Nogo/Nogo-66 receptor signaling pathway involves the transmembrane co-receptors LINGO-1 and p75, the Rho GDP dissociation inhibitor (Rho-GDI), RhoA and its effector Rho kinase (ROCK) and many other effector proteins which, upon phosphorylation, mediate the inhibition for axon growth, regeneration, and structural plasticity in the adult mammalian CNS (Kaplan and Miller, 2003; Brösamle and Halpern, 2008). The Nogo signaling pathway raises a lot of interest as a potential therapeutical target, since *in vivo* neutralization of Nogo activity results in enhanced axonal regeneration and functional recovery following CNS injury (Grandpré and Strittmatter, 2001).

2.9.3 Immune response

Following a tissue injury and subsequent inflammation, the CNS-endogenous cells may themselves initiate, regulate and sustain an immune response. The role of activated microglial cells is well documented in the case of infection, ischemia, trauma, and neoplasia, which elicit a similar inflammatory response in the CNS (Becher et al, 2000). Astrocytes too participate in the immune response. They have been shown to function as intracerebral antigen presenting cell and also have the capacity to express class II major histocompatibility complex (MHC) antigens and costimulatory molecules (B7 and CD40) which are critical for antigen presentation and T-cell activation. They also have the ability to produce a wide array of chemokines and cytokines (Girvin et al, 2002).

2.9.4 Medical considerations

Although the precise contributions of reactive glial cells to neurodegeneration remain controversial, there is mounting evidence that reducing inflammation in the CNS by preventing glial activation could have clinical benefits for patients

suffering from neurodegenerative diseases or traumatic injury. For instance, experiments using heparin have provided promising results for the development of treatments aimed at restoring functional integrity. A single injection of heparin oligosaccharides, given into the wound cavity just after cryo-injury in the cerebral cortex of a mouse, attenuated glial scar formation by decreasing the intensity of astrocytes activation and causing the astrocytic cellular processes to be less branched (Hayashi et al, 2004). Heparin, originally isolated from canine liver cells in 1916, mimics many of the biological activities of abundantly expressed basement membrane heparan sulfate proteoglycans and can even inhibit α-DG binding to laminin (McDearmon et al, 1998). Nevertheless, current medical therapies exhibit limited efficacy in reducing neurological injury and the prognosis for patients remains poor. The development of new strategies and targets to limit neurovascular damage and promote functional recovery after adult CNS injury must rely on the understanding of the underlying biochemistry of scar formation, which can't be achieved without good models to study astrogliosis.

2.10 The *In Vitro* Model for Studying the Glial Scar

Since astrocytes have been relatively difficult to study *in vivo*, the development of *in vitro* models of astrocyte migration has provided a useful tool to investigate the molecular mechanisms controling astrocyte migration. An *in vitro* scratch assay model to study astrogliosis was first developed by Yu *et al* in 1993 and subsequently refined by others (Faber-Elman, 1996; Etienne-Manneville and Hall, 2001, 2003; Perkny, 2005). This system allows the observation of astrocytic response to an injury in a culture environment lacking interactions with neurons. The basis of this model consists of scratching a confluent monolayer of astrocyte grown in controled conditions with either a pipet tip or a blunted small gauge needle in order to simulate an injury after a cerebral mechanical trauma. This experimental procedure initiates a change in the morphology of the cells at the wound edge and recapitulates most of the major astrocyte responses to *in vivo* injury, which is one of the major advantages of the method. Using this assay, we

can therefore simultaneously investigate the mechanisms controling cell polarization, cell protrusion, and cell migration.

Upon creation of the artificial gap made by the blunted needle, the cells on the edge of the newly created gap acquire the reactive phenotype previously described and project a long protrusion perpendicularly to the wound. Cell protrusion is initiated on scratching and stops when the two edges of the scratch meet and cell–cell contacts are established again. Typically, after approximately 24 hours, these protrusions fill the breach and the initial scratch disappears.

The *in vitro* scratch assay is an easy, straightforward and economical method to study cell migration *in vitro* and has been well documented for different cell types. The present study uses this reference model to investigate the role of ECM receptors dystroglycan and integrin in astrocyte wound healing *in vitro*, and proves once again its relevance as a tool for modern fundamental research.

Chapter 3 – Material and methods

3.1 Reagents

FITC-coupled cholera toxin B subunit, 4,6-diamidino-2-phenylindole (DAPI), and antibodies directed against β-tubulin (clone PUB-2.1) and Rac were obtained from Sigma (St. Louis, MO). Antibody directed against α–DG (clone IIH6c, VIA4.1) were obtained from Chemicon (Temecula, CA). The affinity-purified β-DG antiserum was generated in house by inoculating rabbits with a polypeptide corresponding to 15 of the last 16 amino acids of the COOH terminus of ß-DG (Jacobson et al, 2001). The monoclonal antibody 3A3, specific for the alpha(1) subunit of rat alpha(1)beta(1) integrin, has been previously described (Tomaselli et al., 1990). The monoclonal anti-integrin beta(1) antibody was produced in mouse (clone W1B10) and was purchased from Sigma (St. Louis, MO). Cdc42 and Dlg1 (SAP90) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Secondary antibodies were obtained from Jackson ImmunoResearch Labs, (West Grove, PA). Guanosine 5′-[γ-thio]triphosphate (G8634), Cytochalasin D (C8273), colchicine (C9754), retinoic acid, laminin, poly-L-lysine, gelatin, collagen IV and TRITC-conjugated phalloidin were all purchased from Sigma (St. Louis, MO). Fibronectin was obtained from Chemicon (Temecula, CA).

Dulbecco's modified Eagle's medium (DMEM), alpha modified Eagle's medium, fetal bovine serum, horse serum, penicillin/ streptomycin, L-glutamine, pyruvate, nonessential amino acid, trypsin/EDTA, and knockout serum free supplement, were all obtained from Invitrogen (Carlsbad, CA).

3.2 Primary astrocyte cultures

Primary cultures of type 1 astrocytes were prepared from the brains of Sprague–Dawley rats at postnatal day 1 or 2. The meninges, the brain stem and the two

hippocampi were removed under a microscope with sterilized surgical tools. The brain hemispheres were mechanically dissociated in 0,05 % trypsin/ EDTA buffer (Invitrogen Carlsbad, CA) using sterile Pasteur pipettes until a homogeneous extract was obtained. The extract was centrifuged at 1000 rpm for 5 min. The resuspended pellet was seeded on to 175 cm^2 tissue culture flasks pre-coated at least 1 hr with 100 µg/mL of poly-L-lysine or 0.1% gelatin. The brain extracts were incubated at 37^oC, 5% CO2, in astrocyte culture medium (Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% foetal bovine serum (FBS), 100 U/mL penicillin/streptomycin and 2 mM L-glutamine) until the population of the different brain cells reached confluence, approximately after 7 days. The medium was changed everyday for the first 3 days, then every 2 days.

To get rid of other cell types contained in the brain extract (neurons, oligodendrocytes, microglia), the culture flasks were sealed with parafilm and shaked overnight in a regular incubator at 100 to 200 rpm at 37^oC in MEM media + MEM vitamin solution (100x solution from Invitrogen cat. No.11120-052). The next day, the cell monolayer was washed with PBS and the flask tilted gently to remove remaining unwanted cells. Astrocytes remained strongly attached to the extracellular matrix while the other cell types detached.

Astrocytes were then harvested with 5 ml 0,05% trypsin-EDTA and plated onto coated dishes or glass coverslips supplemented with 10% FBS/DMEM for subsequent experiments. For long term storage, the cells were stored in liquid nitrogen in 10% DMSO / 20% FBS/DMEM (2E6 cells/ml).

3.3 ES cell-derived astrocyte culture

The protocol for the differentiation of embryonic stem cells into astrocytes was developed by our research group and has been published in Peng *et al*, 2008. DG null and beta(1) integrin null ES cells were obtained as previously described (Fassler et al, 1995; Cote et al., 1999).

Embryonic stem (ES) cells in differentiation medium (DMEM High-Glucose, 15% Horse Serum, 1% Pen-Strep) were seeded at a density of 5 X 10^5 cells/mL, using a multichannel pipettor, as 20 μL drops onto the inside of the lid of a 100 mm diameter plastic Petri dish. In order to form embryoid bodies, the ES cells were cultured at 37^0C in a 5% CO_2 incubator as hanging-drops for 4 days. Retinoic acid was added into the culture medium to a final concentration of 5 μM and the embryoid bodies were cultured for another 4 days.

The embryoid bodies were then transferred onto dishes pre-treated with a solution containing 100 μg/mL poly-L-Lysine and 10 μg/mL laminin and were cultured in neuron differentiation medium (MEM alpha medium, 5% knockout serum free supplement, 20 mM glutamine, 10 mM pyruvate, 1 mM nonessential amino acids, 1 mM β-mercapto-ethanol).

When neuron-like cells appeared from the embryoid bodies, the culture medium was changed to astrocyte differentiation medium (MEM alpha medium, 5% knockout serum free supplement, 20 mM glutamine, 10 mM pyruvate, 1 mM nonessential amino acid, 10 mM β-mercaptoethanol, 20 ng/mL LIF (leukemia inhibitory factor)).

The differentiated embryoid bodies were dissociated, cultured in astrocyte differentiation medium, and the differentiated astrocytes were purified by overnight shaking to remove unwanted cell types. The purified astrocytes were then harvested and plated onto pre-treated dishes or glass coverslips and used for the microsomial fractionation to detect DG and the Cdc42 wound assay.

3.4 Microsomal extraction of DG

Astrocytes were cultured in 100 mm culture dishes coated with 0,1% gelatin. After the cultures were confluent, cells were detached with 15 mM sodium citrate, 150 mM KCl, 1mM EDTA for 5 min and washed once with ice-cold PBS containing 1X protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Cells were homogenized in 0,32 mM sucrose, 10 mM Hepes (pH 7.0) 150 mM NaCl, 0.5 mM Ca2Cl, 1X protease inhibitor cocktail. Homogenates were centrifuged at 1000g for 10 min at 4^{o}C to obtain the pellet (P1) and microsomal fraction (S1). The microsomal fraction was then centrifuged at 200,000g for 2 h in a Beckman TLS-55 rotor to obtain the microsomal pellet (P2) and soluble fraction (S2). Fraction P2 was solubilized with buffer (10 mM Hepes (pH 7.0) 150 mM NaCl, 0.5 mM Ca_2Cl, 1X protease inhibitor cocktail) containing 1.0% Triton-X 100. Proteins were electrophoresed in 8 or 12% SDS-PAGE and transferred to nitrocellulose membrane 2 hr at 100 V, 4^{0}C. Membranes were blocked overnight in 1% casein dissolved in TBS buffer, then probed with IIH6 and β-DG antibody using appropriate HRP-labeled secondary antibodies. A skeletal muscle extract, obtained the same way from myotubes cultures, served as a positive control.

3.5 Adhesion assay

The glass coverslips were coated with a solution of the adhesion molecule of interest in 24-well cell culture plates for at least 1 hr after which they were rinsed with PBS. Astrocytes in culture were released with trypsin and transferred to the coated areas at a density of 2500 cells/ sample with freshly harvested P1 astrocytes. Cells were incubated at 37^{o}C, 5% CO_2 in 10% FBS/DMEM. After specific time points, cells were washed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} PBS and fixed with 4% para-formaldehyde (PFA) dissolved in PBS for 10 minutes. Coverslips were maintained in PBS at 4^{o}C until they were used for immunofluorescence studies.

3.6 Immunofluorescence microscopy

Astrocytes were seeded on pre-treated coverslips, coated with the ECM molecule of interest, in 24-well culture plates. (The round glass coverslips had been previously sterilized with nitric acid overnight, rinsed 10 times with dH_2O, followed by overnight soaking in 100% ETOH and air dried under a sterile flow hood.) Culture media was removed and cells rinsed once with room temperature PBS. Cells were fixed with 4% PFA dissolved in PBS for 10 minutes. PFA was washed away twice with PBS and all cell plasma membranes were permeabilized with fresh 0.25% Triton X-100/PBS for 10 min. Samples were washed twice with PBS, then blocked 1 hr with 10% Horse serum (HS) / PBS. Incubation with primary antibody was carried out overnight for 1 hr at RT in 5% HS/PBS. After three 10 min washes with PBS, fluorescent dye-conjugated secondary antibodies diluted in 5% HS/PBS were applied for 1 hr at RT, covered from light, followed by three 5 min washes with PBS. Incubation with the secondary antibody alone served as negative control for background evaluation.

To stain nuclei and actin fibers, cells were incubated for 10 min at room temperature with DAPI and fluorescent dye-conjugated phalloidin, respectively, in PBS. To stain GM1, cells were incubated at room temperature with cholera toxin B-FITC / PBS for 15 min, followed with a PBS wash. After the antibody staining procedure, the coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlington, ON). Images were captured using a Qimaging Retiga 1300 10-bit digital camera mounted on a Zeiss Axioskop fluorescence microscope (Zeiss, Germany), using the 63x objective, and were processed using Northern Eclipse Version 7.0 image analysis software (Empix Imaging, Mississauga, ON, Canada).

3.7 *In vitro* wound assay

This assay was adapted from Etienne-Manneville *et al*, 2001. A suspension of astrocytes (2,5 x 10^5 cells/ml) was seeded on to pre-treated 1D round glass coverslips in 24-well culture plates and incubated in astrocyte culture medium at

37°C, 5% CO2, until they reached confluence. Cells were washed three times with PBS and were wounded by scraping across the monolayers with a blunted 18G needle, to form wounds ~300 µm in width. Cells were fixed with 4% PFA dissolved in PBS, at specific time points and used for immunofluorescence purposes.

3.8 Cytochalasin D and colchicine treatment

Astrocytes (1 mL of a suspension of 2,5 x 10^5 cells/ml) were seeded on to glass coverslips (pre-treated with a solution of laminin 100 µg/ml) and incubated at 37°C, 5% CO_2 in astrocyte culture medium, until they reached confluence. The cells were washed once with 37°C PBS and new media was added with 0,25, 5 or 1 µM of cytochalasin D, or with 5 or 12,5 µg/ml of colchicine. Astrocytes were immediately wounded by scraping across the monolayer with a blunted 18G needle and the cells remained in same media until fixation. After 4 and 8 hr of incubation, cells were fixed with 4% PFA dissolved in PBS and stained with Coomassie blue. Photomicrographs were taken at 200x magnification. For each conditions, the length of protrusions of 100 cells at the leading edge of the scratch-wound were measured from the top of the nuclei to the tip of the protrusion perpendicularly to the scratch, using Northern Eclipse Version 7.0 image analysis software (Empix Imaging, Mississauga, ON, Canada).

3.9 Cdc42/Rac activation assay

Generation of stock GST and GST-CRIB bacterial clones:

Vials of BL21 competent *E. coli* transformed with the plasmid of either GST or GST-PAK-CRIB (crib is the Cdc42 binding domain of p21 activated kinase -PAK) were a kind gift from Dr. Nathalie Lamarche (McGill University). Bacteria were seeded on Agar plates with appropriate selection markers and grown overnight at 37°C. A colony was picked for each clone the next day and cultured overnight at 200 rpm, 37°C in a 14 ml polypropylene round-bottom flacon tube filled with 5 ml of LB amp+. GeneElute Five minute plasmid Miniprep kit from Sigma (PFM10) was used to extract the plasmids which were then linearized with Xho1 for

genetic verification. DNA concentration was assessed by running an electrophoresis on a 1% agarose gel along with λ/Hind III DNA. Stocks for each strain were kept at -80^{o}C.

Induction of GST and GST-PAK-CRIB expression:

In order to induce the expression of the two recombinant proteins, the bacterial strains were cultured at 37° in 1 L of LB medium containing 50 µg/ml ampicillin to an OD_{595} 0.2–0.3. After the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 m*M*, bacteria were further cultured for 4 h to induce the production of GST and GST-PAK-CRIB. Bacteria were harvested by centrifugation at 5,000 rpm at 4° for 5 min, the supernatant was discarded, and the bacterial pellet was washed with 20 ml ice-cold PBS. The suspension was centrifuged at 5,000 rpm at 4° for 5 min and the supernatant discarded. All subsequent procedures were performed at 0–4°C. The pellet was suspended with 6 ml of ice-cold lysis buffer A (20 mM Hepes pH 7.5, 120 mM NaCl, 2 mM EDTA, 10 % glycerol, protease inhibitor aprotinin, leupeptin and 1 mM PMSF). Lysed bacteria suspensions were sonicated at mid-speed by Ultrasonic Processor (Taitec, Tokyo, Japan) on ice for 15 sec, four times, at 30 sec intervals. The homogenate was centrifuged at 10 k, at 4°C for 15 min and the supernatant was purified with affinity chromatography.

Affinity Purification of GST and GST-PAK-CRIB:

Glutathione-sepharose 4B (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) beads are packed onto a 10-ml disposable syringe (bed volume, 1 ml). The beads were washed with 20 ml of PBS twice and equilibrated with 20 ml of PBS. 20 mL of crude supernatant prepared as previously described was applied to the syringe column, and the eluate was reapplied to the column. After the column was washed with 20 ml of PBS, GST or GST-PAK-CRIB was eluted with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8,0). The fusion proteins were kept as purified recombinant GST and GST-PAK-CRIB at −80° for at least 6 months without loss of activity.

Binding assay:

Prior to the lysis of astrocytes, 60 µg of recombinant GST and GST-PAK-CRIB were coupled to 50% slurry Glutathione Sepharose beads in lysis buffer A for 60 min at RT on a rotator. Three different amounts of protein (20, 40 and 60 ug) were tested and we found that 60 ug of coupled fusion protein gave the best results.

Astrocytes were grown to confluence in 15 cm in diameter culture dishes coated with laminin (100 µg/ml). Prior to scratching, cells were synchronized overnight (o/n) with 2% FBS/DMEM. A cross rule of 15 rows and columns was made with a blunted 18G needle in order to wound the cells. After 5 or 15 min, the cells were washed three times with PBS without Ca^{2+} and Mg^{2+} before they were scraped off with a rubber policeman in PBS. A confluent monolayer of cells, unscratched, was harvested the same way and considered as T=0. The cell suspension was centrifuged at 3500 rpm, 5 min and the pellet resuspended in 1.5 ml of lysis buffer B (25 mM Hepes pH 7,5, 10 mM MgCl2, 100 mM NaCl, 1% NP-40, 5 % glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM PMSF and 1 mM sodium vanadate). The suspension was incubated 10 min on ice with occasional pipet trituring and inversion. The lysate was cleared with a 10 min centrifugation at 4^{o}C at 13 K and filtered using a sterile 0,2 µm filter. Samples of the cleared lysates were kept to determine protein concentration. Equal amounts of proteins, or 250 µl of protein lysate, were applied to the coupled fusion proteins in 2 volumes of binding buffer (25 mM Hepes pH 7,5, 30 mM $MgCl_2$, 40 mM NaCl, 0,5 % NP-40 and 1 mM DTT) and incubated on a rotator for 60 min at 4^{o}C. Guanosine 5′-[γ-thio]triphosphate (GTP-γ-S) is a non-hydrolyzable GTP analog and was used as positive control for the assay (at 10 mM). GST-coupled beads alone, GST-PAK-CRIB-coupled beads alone, uncoupled beads incubated with lysate, GST-coupled beads incubated with lysate, GST-PAK-CRIB incubated with lysate and GDP (100 mM) served as negative controls.

The beads were then washed 3x with wash buffer (25 mM Hepes pH 7,5, 30 mM MgCl2, 40 mM NaCl and 1 mM DTT) containing 1% NP-40 and 2x with wash buffer alone. The beads were re-suspended in 10 µl 2x Laemmli sample buffer (with 100 mM DTT), boiled 5 min and centrifuged. The resulting supernatant (15 µl), along with equal amount of proteins (or 15 µl) of total lysate were loaded on a 12 % protein gel, transferred on to nitrocellulose membrane and blot with rabbit polyclonal Cdc42 antibody (1:450). GTP-bound Rac can also be detected with the same GST fusion proteins, since Rac recognizes the Crib domain of Pak as well. Thus, the same membrane was stripped with 0,2 M glycine pH 3 for 15 min, washed with TBS and then blot for Rac1 (1:800). Blots were developped using goat anti–mouse (for Rac1) or goat anti–rabbit (for Cdc42) coupled to horseradish peroxidase (1:10,000 dilution) and visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). The film (Kodak Biomax from Sigma) was developed after a 15 min exposition.

Chapter 4 – Results

4.1 Dystroglycan expression in adherent primary rat astrocytes

The DG subunits are expressed in primary rat astrocytes. The molecular weights of α-DG and β–DG were approximately 120 and 43 kDa, respectively (Fig.1). The skeletal muscle positive control has a higher molecular weight than brain DG, which is as expected.

In this study, we use the term adherent astrocyte, as opposed to migrating astrocyte. We observed that non-stimulated, adherent astrocytes adopted 4 different basic morphologies depending on the time they were allowed to spread. These spreading stages were termed as astrocyte spreading stages 1 to 4. They occurred independently of the ECM used to coat the glass coverslips on which the astrocytes were cultured (fibronectin, collagen or laminin). Astrocytes attachment to the matrix also occurred in serum free medium, but was much improved by addition of serum to the medium, which has been the case for all the results presented here. The observation of the 4 stages led to the hypothesis that the distribution of dystroglycan would vary with regard to the spreading stage on laminin. To assess this idea, the distribution of β–DG was characterized by immunofluorescence in astrocytes allowed to spread 30 min, 4h, 8h and 24h. The cells were then fixed with 4% PFA and stained with the polyclonal β–DG antibody.

At stage 1, the cells looked like small spheres with no visible protrusion. This stage of adhesion could be fully appreciated 30 min after seeding the cells on to matrix-coated glass coverslips. β–DG was localized centrally and in nascent adhesion structures around the cell periphery (Fig. 2A).

At stage 2, the cells began to spread and protrusions were formed as well as defined adhesion structures, especially point contacts. This stage was usually reached 1hr after seeding or sooner and visible up to 4hr. Multiple visible adhesion structures containing β–DG appeared around the cell periphery (Fig. 2B).

Astrocytes at stage 3 started to flatten and numerous filopodia and lamellipodia were visible, with a trend to form mainly a lamellipodial cell edge, rather than an increase complexity of stellation. Spreading stage 3 could be observed at least 1h after seeding the cells. The three first spreading stages coexisted within the first two hours, but stage 3 became predominant between 4hr and 8hr. Focal contacts could be clearly visualized in the third stage of adhesion (data not shown). β–DG was still localized to the tip of extending process but less clustered and there seemed to be a diffused pool of β–DG forming in the cytoplasm (Fig. 2C).

Astrocytes in the fourth and final spreading stage adopted a wide polygonal and flat shape with almost uniform edges, reminiscent of well spread fibroblasts. This spreading stage could be observed clearly at least four hours after seeding, while stage 2 became less frequent and stage 1 almost completely disappeared. After 24hr, most cells had reached stage 4. The distribution of β–DG at the cell edges became diffuse and weak while a cytoplasmic pool of β–DG had formed perinuclearly (Fig. 2D). At low cell density, stage 4 astrocytes do not tend to migrate. They rather firmly adhere to the matrix and slowly divide, eventually covering up the whole surface.

4.2 Distribution of dystroglycan and integrins with lipid rafts in adherent astrocyte

In many cell types, dystroglycan has been associated with numerous signaling molecules complexed at the membrane and involved in cytoskeletal reorganization, as described in chapter 2. In embryonic stem cells, DG was not associated with GM1-rich lipid rafts extracted by sucrose gradient centrifugation (Shah et al, 2006). Still, since integrins have been shown to colocalize with lipid rafts in adherent fibroblasts (del Pozo et al, 2004), I verified if DG would colocalize with the lipid raft marker GM1 in adherent astrocytes. The B subunit of cholera toxin (CT-B) selectively binds to the ganglioside GM1 and has been used widely to localize lipid rafts in cells (Parton, 1994). In order to address this matter, the distribution patterns of α–DG and GM1 in adherent astrocytes cultured on laminin were compared using immunocytochemistry.

The results suggest a very low degree of colocalization for the two molecules at the plasma membrane level. There was higher colocalization in what seemed to be an intracellular pool of both molecules around the perinuclear region. The distribution pattern of most DG clusters (short arrows) at the cell edge did not fit any corresponding GM1 clusters (long arrows). In the space between the plasma membrane and the perinuclear space, α–DG and GM1 were barely detected (Fig. 3). Overall, the distribution of α–DG is more discrete than GM1, which has a more continuous pattern. It cannot be excluded that α–DG and GM1 codistributed in some discrete regions of the cell edge, but nothing comparable to the colocalization of beta(1) integrin and GM1 (Fig. 4F) for the same spreading stage.

Integrins are known to initiate their transduction signals from GM1-rich lipid rafts and both $\alpha 3\beta 1$ and $\alpha 5\beta 1$ integrins mediate astrocyte adhesion on fibronectin (Humphries et al, 2006). It was hypothesized that the codistribution of GM1 and

beta(1) integrin would evolve as the astrocytic cell changes to more advanced morphological state of adhesion. To address this hypothesis, the distribution of beta(1) integrin and GM1 was monitored by immunofluorescence at stages 1 and 2. At spreading stage 1, the distribution of the beta(1) integrin and the lipid raft marker GM1 was diffuse and more concentrated in the central region of the cell (Fig. 4A-C). As the cell spread and its adhesion structures developed, beta(1) integrin seemed to get recruited more to the cell periphery where it colocalized with GM1 at the cell edge (Fig. 4D-F). At a more advanced state of stage 2, the beta(1) integrin subunit clustered into radial and elongated structures which could possibly be focal contacts, according to their thin and elongated morphology, and GM1 seemed almost absent from these structures (Fig. 4G-I). These data suggest that indeed, the codistribution of beta(1) integrin and GM1 varies with the cell spreading stage.

4.3 Distribution of alpha(1)beta(1) integrin and Dlg1 in adherent astrocytes

It has been established by our laboratory that type 1 astrocytes, but not oligodendrocytes or type 2 astrocytes express the alpha(1) and beta(1) integrin heterodimers (Tawil et al, 1994). The clear signal from the 3A3 antibody in figure 5, specific to the alpha(1) subunit, provides an evidence to demonstrate that the GFAP expressing primary rat astrocyte subpopulation, used throughout the project, presented a high degree of type 1 homogeneity.

The tumor suppressor Dlg1 is essential for the assembly of multiprotein complexes at cell-cell junctions and is involved in maintenance of cell adhesion in epithelial cells (Humbert et al, 2003). Dlg1 activity has been linked to cell polarization signaling during astrocyte migration (Etienne-Manneville et al, 2005; Osmani et al, 2006), but there is no clear evidence of a role for Dlg1 in astrocyte adhesion. Our laboratory has shown that the beta(1) integrin subunit plays an important role in astrocyte adhesion, polarization and migration (Peng *et al*,

2008). Taken together, these data led to the hypothesis that Dlg1 might be involved in alpha(1)beta(1)-mediated adhesion. The distribution patterns of the alpha(1) integrin subunit and Dlg1 in freshly attached astrocytes on laminin, were investigated using immunocytochemistry.

The alpha(1) integrin subunit distributed mainly to the edges of the numerous lamellipodia all around the peripheral plasma membrane, clustered to adhesion sites. It did not colocalized with the tumor suppressor Dlg1. Instead, Dlg1 appeared diffuse in the perinuclear region of the cytoplasm and was almost completely absent at the cell edges. This suggests that Dlg1 is not involved in alpha(1)beta(1) integrin-mediated adhesion in non-stimulated, adherent astrocytes grown on laminin.

4.4 Cytoskeletal characterization of reactive astrocytes

The protrusions of reactive astrocytes were mainly filled with long arrays of microtubules, while actin bundles extended in the direction of protrusion but remained behind, at the level of the original cell edge made by the scratching needle (Fig. 6). The two cell edges from each side of the scratch came together approximately 16 to 24 hours later, leaving basically no trace of the initial scratch (data not shown). Primary astrocytes that have been in culture for a longer period of time (2 or 3 weeks), grow slower and will often take more than 24 hours to fill the breech. In our model, when the two cell edges from each side of the scratch come together and fill the gap, this is considered as the final step of glial scaring.

Based on our own observations (Fig. 6) and the literature, microtubules seem to be mainly responsible for protrusion formation. This raised the question whether cytochalasin D, an actin cytoskeleton disrupting agent, would affect protrusion formation in reactive astrocytes. In order to address this matter, freshly scratched monolayers of primary astrocytes were treated with different concentrations of cytochalasin D (0.25, 0.5 and 1 µM) and the length of protrusions of the leading

edge reactive astrocytes were measured 4 and 8 hours after scratching. Compared to controls, which reached over 120 and 160 µm, at 4 and 8 hr respectively, cytochalasin D partially inhibited protrusion formation at all concentrations used in the experiment (Fig. 7A). The effect of the drug persisted over time, as protrusion lengths were smaller at 8 hr than at 4 hr after scratching. The three concentrations had a similar effect for the 4 hr time point. The effect on protrusive activity was the greatest with 1 µM of cytochalasin D after 8 hours.

Colchicine is a drug known to inhibit the polymerization of MTs by preventing the assembly of tubulin dimers. It was expected that this drug would inhibit protrusion formation in astrocytes and we compared its effects to the ones of cytochalasin D. The lengths of protrusions were measured 4 and 8 hours after scratching monolayers of primary astrocytes treated with 5 or 12,5 µg/ml (12,5 and 31,3 µM) of colchicine. As expected, colchicine inhibited process extension (Fig. 7B). The effect of colchicine was greater at 4 hr than 8 hr. At the 4 hr time point, a concentration of 12,5 µg/ml of colchicine almost disrupted the cell monolayers and the cell edges started to ruffle (data not shown). No measurements were possible at that concentration, thus the lengths of protrusions were considered as zero. It was concluded that both cytochalasin D and colchicine inhibit protrusion formation. Thus, even though protrusion formation in reactive astrocytes seemed to be a MT-driven process, it is not actin-independent.

A fine line of cortical actin at the tip of extending processes in reactive astrocytes was first observed by Etienne-Manneville and colleagues (Etienne-Manneville et al, 2001). This observation was confirmed with a double immunostaining for both actin and MT cytoskeletons, in reactive astrocytes extending processes perpendicularly to a scratch (Fig. 8A). The cortical actin actually surrounded the cell edge completely and did not seem to be linked to the actin bundles that remained at the rear of the protrusion. Since the protrusions were mainly filled with long arrays of microtubules, we hypothesized that cortical actin formation at the tip of reactive astrocyte protrusions could be a MT-dependent process. In

order to verify this hypothesis, astrocyte monolayers were treated with the same concentrations of colchicine as in Fig. 7 and both actin and MT cytoskeletons were visualized by dual immunofluorescence.

The actin network still produced actin bundles after treatment with colchicine at 5 or 12,5 µg/ml (Fig.8B-C). The MT network, on the other hand, appeared scrambled and disorganized compared to the control. A magnification of Fig. 8C showed that even at a concentration of 12,5 µg/ml, that partially inhibited protrusion formation and disrupted the polarized MT network, colchicine did not prevent cortical actin localization at the cell edge (Fig. 8D). This suggests that cortical actin localization in reactive astrocytes is independent of the polarized MT network.

4.5 Immunolocalization of ECM receptors and lipid rafts in migrating astrocytes

Our laboratory published the first evidences that revealed a role for dystroglycan in astrocyte proliferation *in vitro* (Peng et al, 2008). I present here complementary data that support these findings. Figure 9 shows the subcellular localization of DG subunits with respect to both actin and MT cytoskeletons in reactive astrocytes, 8 hours after the initial scratching of the monolayer. α–DG was found at the tip of the protrusions, as well as along the axis of the extending process (arrows, Fig. 9A). β–DG was observed at the tip of the extending process, slightly ahead of the MT-rich protrusion and possibly in association with cortical actin (arrows, Fig. 9E). DG was also found behind the MT-rich region in the core of the cell. Other preliminary observations suggest that the distribution of the DG subunits may be time-dependent in migrating cells (Peng et al, 2008), as it is the case for non-stimulated, adherent astrocytes (Fig. 2).

A high signaling activity in microdomains at the leading edge of reactive astrocytes has been reported by numerous research groups. Proteins recruited to these organizational platforms can modify their phosphorylation state by means of raft-associated kinases and phosphatases resulting in downstream signaling (Simons and Toomre, 2000). Based upon our own preliminary results and the literature, it was hypothesized that when astrocytes become reactive, the signaling machinery, including lipid rafts and the ECM receptors integrin and dystroglycan, is targeted to the tip of protrusion to lead process extension. Here, some evidences are provided showing that both DG and GM1 distributed at the leading edge of the extending process of a reactive astrocyte (Fig 10A-B), but the sampling does not allow to conclude that the two molecules colocalized. The integin subunit alpha(1) also localized to the cell edge, although the cell depicted in Fig.10C is different than in Fig.10A and B.

4.6 Dystroglycan and Cdc42/Rac activation in wounded astrocytes

Etienne-Manneville and colleagues had previously shown that the Rho GTPase Cdc42 was activated in minutes after wounding a monolayer of primary astrocytes (Etienne-Manneville et al, 2001). The results presented here support their findings. In a Cdc42/Rac activation assay, 250 µl of cell lysate were incubated with 60 µg of coupled GST fusion proteins at three different time points and the resulting final supernatants, along with 15 µl of total lysate, were loaded on a 12 % gel. The amount of total lysate proteins loaded on the gel for T=0, 5 and 15 min was 41.1, 58.4 and 41.9 µg, respectively. The amount of total lysate proteins incubated with the coupled GST fusion proteins for T=0, 5 and 15 min was 681.8, 974.0 and 698.1 µg, respectively. Activated Cdc42 was detected at T=0 min, but the level of activation significantly increased as soon as 5 minutes after wounding and was maintained for at least 15 minutes (Fig. 11A). The same membrane used for Cdc42 western blotting was stripped and then blot for Rac, another Rho GTPase that has an important role in astrocyte migration. Activated

Rac was detected at T= 0, but the signal intensities seemed to be increased for T= 5 and T= 15 min. These results suggest that Rac activity was also increased early after wounding but we can't conclude since not equal amounts of proteins were used to perform the assay.

Our laboratory had previously developed an embryonic cell line null for DG (Côté et al, 1999). One the goals of the project was to derive an astrocytic cell line from these embryonic stem cells in order to study the role of DG in Rho GTPases signaling. Two astrocytic cell lines were successfully developed, one null for DG, and the other was wild type for DG (Fig. 12). Another ES cell-derived astrocytic cell line null for beta(1) integrin was also developed. Once fully differenciated and purified, these cells expressed GFAP and were cultured the same way as the regular primary astrocytes. Their growth rate was slightly faster than primary astrocytes, even for the wild type ES cell-derived astrocyte cell line. Moreover, we observed that these three cell lines were useful for experiments, such as adhesion or scratch assays, only in a relatively short period of a few days. Beyond that window of time, undifferentiated ES cells would grow back on top of the astrocyte monolayer and within 3 or 4 days, these small and round ES cells would cover the whole monolayer, rendering the astrocytic cells underneath useless to work with.

Chapter 5 - Discussion

DG and integrins in astrocyte adhesion

Dystroglycan and the integrins are broadly expressed in the CNS and function as adhesion receptors. In the present study, the role of the two main astrocytes ECM receptors was investigated. We show that both types of receptors are differentially distributed to the plasma membrane, depending on the cell spreading stage. Integrins are already known to signal from GM1-rich lipid rafts via the Rho GTPases signaling pathway to control cytoskeletal organization. On the other hand, the contribution of DG in the control of astrocyte behaviour is much less understood. This study tries to shed light on the underlying mechanisms controling the distribution of the two ECM receptors in astrocyte adhesion and migration.

Adherent astrocytes cultured without stimulation on a basic matrix adopt a sequence of morphologic events and four basic morphologies emerged from our observations. Since several morphological stages can coexist at a given time point, I found that it is more exact to compare adherent astrocytes between their morphological stage rather than spreading time when immunocytochemistry is the technique of choice. In addition to the four different basic morphologies described in section 4.1, astrocytes can change to a more stellated shape, which resembles more their *in vivo* appearance, when stimulated with cAMP agonists, or simply by replacing the culture medium with saline buffered with HEPES (Cechin et al, 2002). These stimuli initiate a change termed stellation characterized by a process-bearing morphology accompanied by a loss of actin stress fibers and focal adhesions. RhoA seems to be in control of this astrocyte morphology, since astrocytes expressing activated RhoA fail to undergo cAMP-induced stellation, while inactivation of RhoA is sufficient to induce stellation (Ger et al, 1998; Abe et al, 2003).

In the DG subcomplex, α–DG is the most heavily glycosylated subunit, with a molecular mass of approximately 74 kDa for the core protein, which can reach up to 180 kDa in certain tisues after post-translationnal modifications. Due to tissue-specific differential glycosylation, skeletal muscle α–DG has a higher molecular weight than astrocyte DG, typically 156 kDa (Ibraghimov-Beskrovnaya et al, 1992). DG is also expressed in populations of neurons throughout the brain, including the cerebral cortex, olfactory bulb, hippocampus, basal ganglia, thalamus, hypothalamus, brainstem and cerebellum (Zaccaria et al, 2001).

The subcellular localization of β–DG varied with the astrocyte spreading stage. The shifting from the distal tips of protrusions to the central part of the cytoplasm in the distribution pattern suggests that β–DG might be important for the formation of attachment points in early spreading stages, assuming that it remains associated with α-DG. Other ECM receptors, possibly beta(1) integrins, may take the relay as the cell flattens and enters stage 4. Nevertheless, research from our laboratory has shown that DG is an important mediator of astrocyte adhesion (Peng et al, 2008) and the data presented here support these conclusions.

Many integrins are responsible for cell adhesion on fibronectin (α3β1, α5β1, α4β1, αvβ1, α8β1, α4β7, αvβ6, αvβ8, αvβ3 and αIIbβ3) and among them, both α3β1 and α5β1 are known to mediate astrocyte adhesion (Takada et al, 1991; Tawil et al, 1993; Humphries et al, 2006). From our observations, beta(1) integrins effectively got recruited to focal adhesions as the cell spread on the ECM. Indeed, the radial and elongated structures observed in Fig. 2G-I match the description of focal contacts associated to integrin signaling described in other cell types (Webb et al, 2002). A double immunostaining with FAK/beta(1) integrin could provide more evidence to support this idea. The different immunofluorescence patterns, for dystroglycan and integrin in spreading astrocytes, strongly suggest that the subcellular localization of both ECM receptors is dependent of the cell spreading stage.

ECM receptors and lipid rafts in adhesion and migration

Lipid rafts are specialized microdomains that provide a matrix for protein support and play an active role in signal transduction and regulation of membrane trafficking (Blank et al, 2007). In the rat astrocyte cell lines used for this project, GM1-rich lipid rafts were identified in low-density, non-soluble fractions isolated with a sucrose gradient centrifugation from confluent monolayers of astrocytes (Carbonetto *et al*, *unpublished results*).

GM1 has been shown to be present in endocytic organelles, in the trans-Golgi network, and in the plasma membrane (Parton, 1994). GM1 localization to the membrane is adhesion-dependent. GM1-rich lipid rafts get internalized in non-adherent cells and translocate back to the cell membrane surface upon adhesion through an endocytic recycling mechanism that appears to be regulated by FAK (del Pozo et al, 2004). The data presented in the present study suggest that GM1 was not distributed uniformly over the plasma membrane. The degree of colocalization with GM1, in non-migrating, adherent astrocytes, was significantly higher with beta(1) integrin than with α–DG. In fact, α–DG barely colocalized with GM1 at cell edges, but mostly to more central regions of the cell. Yet, both α–DG an beta(1) integrins are involved in astrocyte adhesion (Peng et al, 2008) and both cells depicted in Fig. 3 and Fig. 4D-F were at spreading stage 2.

A question that arises then is why these two ECM receptors do not distribute with GM1 similarly? It has been shown that integrins use GM1-rich lipid rafts to target Rho and Rac to the plasma membrane and couple them to downstream effectors of the integrin signaling pathway. In fact, cell detachment causes the loss of Rac membrane targeting and integrin signaling (del Pozo et al, 2004). In fibroblasts, Palazzo *et al* reported that the localized microtubule stabilization at the leading edge of migrating cells is mediated by integrin signaling, which regulates the local coupling of Rho to its effector mDia by the localization of GM1-rich lipid rafts at the leading edge (Palazzo et al, 2004) .

In the case of dystroglycan, it is less clear if DG is recruited to GM1-rich lipid rafts. For instance, in mouse embryonic stem cells, DG was not found associated with GM1-rich lipid rafts but was still associated with cholesterol-rich microsomal extracts (Shah *et al*, 2006). Interestingly, data from another research group, Ambrosini *et al,* published that DG, synthrophin and caveolin-1 were co-extracted in astrocytic detergent-resistant membranes (DRMs)/lipid raft microdomains (Ambrosini et al, 2008). Caveolin is involved in internalisation of GM1. In fibroblasts, when cells were detached and integrin signaling inactivated, caveolin-1 showed time-dependent movement from the plasma membrane to an intracellular compartment on the same time scale as the raft marker GM1 (del Pozo et al, 2005). In the paper of Ambrosini *et al*, however, they did not show if GM1 was present in the DRM extracts they analyzed.

Thus, DG may indeed be associated with a subclass of cholesterol-rich microdomains that contains caveolin-1, but not the ganglioside GM1. The same experiment as in Ambrosini *et al*, but including GM1 blottting, could be repeated to clarify the issue. The present study provides evidence that alpha(1) integrin, β–DG and GM1 are localized at the front edge of the migrating astrocyte. It is possible that, in the case of adhesion, the lipid environment at the plasma membrane may differ for dystroglycan and integrins, but in migrating astrocytes, however, when specific transduction pathways activate the redistribution of lipid raft components, a subset of the DG pool could translocate to GM1-rich microdomains.

This project opens the way to more in depth studies to validate this hypothesis. Further investigations are needed to determine if DG physically interacts with GM1-rich lipid rafts at the tip of reactive astrocytes protrusions. Detergent-resistant membrane fractions could be isolated from non-scratched versus multi-scratched astrocyte monolayers and western blotted for DG, GM1 and other lipid raft markers. More thorough immunofluorescence studies could be performed as well to study the potential recruitment of DG to lipid rafts in reactive astrocytes.

Integrin α1β1 signaling in astrocytes

Wounding an astrocyte monolayer induces several cascades of signals that lead to the redistribution of specific signaling molecules to the plasma membrane. For instance, integrin-mediated activation of the Par6–PKCζ complex by Cdc42 at the leading edge of migrating cells promotes both the localized association of APC with microtubule plus ends, and the assembly of Dlg1-containing puncta to spatially restricted region of the leading edge, essential for the reorganization of the microtubule network (Etienne-Manneville et al, 2005). Moreover, Dlg1 and APC can be co-immunoprecipitated within 1 hr after scratch-induced cell migration, but not in confluent, non-migrating primary astrocytes (Etienne-Manneville et al, 2005). Our results complement and support these conclusions. The signaling pathway regulating Dlg1 recruitment to the membrane seems to remain inactive in non-migrating astrocytes, since Dlg1 is not found to the basal plasma membrane but remains in an intracellular pool. Dlg1 has been involved in maintenance of cell adhesion in epithelial cells (Humbert et al, 2003), but it does not appear to be involved in α1β1 integrin-mediated astrocyte adhesion, at least for early cell spreading stages.

It would be interesting to deepen the analysis of the cellular distribution of integrins and Dlg1 in order to identify precisely which integrin is/are responsible for the initial polarization signal in migrating astrocytes on laminin. Moreover, it would be helpful to determine if they colocalize at the tip of protrusions. The results presented in Etienne-Manneville and Hall, 2001 were obtained on poly-L-ornithine as the matrix and suggest it is an RGD-dependent integrin. As our results showed, α1β1 integrin, which is RGD- independent, is targeted to the tip of protrusions in migrating astrocytes. In addition, migration on laminin can be blocked by a specific function blocking antibody against the α1 integrin subunit (Peng et al, 2008). The issue remains open regarding the effect of the same antibody on polarization and Dlg1 recruitment to the membrane, but it is tempting

to hypothesize that blocking integrin $\alpha 1\beta 1$ function on laminin would perturb Dlg1 relocalization to the membrane in scratch-induced polarization of astrocytes.

Cytoskeletal dynamics in reactive astrocyte

Migrating astrocytes are morphologically different from immobile cells. *In vitro*, they are very flat and firmly adherent. Upon scratching, the leading edge protrudes continuously for at least 12 to 16 hr, forming a MT-rich elongated protrusion extending perpendicular to the wound and reminiscent of the large astrocytic processes visible *in vivo.* Cells do not move individually but rather migrate as a sheet toward the other edge of the scratch.

Cytochalasins, which are fungal metabolites, bind actin subunits and thus prevent their polymerization. According to the reference paper of Etienne-Manneville and Hall, published in Cell in 2001, which was the basis of the present study: “Although cytochalasin D completely inhibited cell migration, it did not prevent the formation of protrusions or the polarization of the MTOC (data not shown).” The results presented here do not fully agree with these findings. We have characterized the cytoskeleton of reactive astrocytes, combining immunocytochemistry with cytochalasin D and colchicine treatments in scratch assays. We found that, while microtubules seem to be mainly responsible for the elongation of the protrusion, this process is not actin-independent. In fact, elongation could be inhibited by both cytochalasin D and colchicine, although through different mechanisms. We also found that cortical actin localization is independent of the polarized MT network. We did not address the issue of polarization in this study, but it was shown that Golgi polarization is actin-independent in reactive astrocytes (Magdalena et al, 2003).

Contrary to cytochalasin D, the effect of colchicine was greater at 4 hr than 8 hr. It seemed that the cells were recovering better after the 8 hr treatment. Colchicine may have been metabolized over time by enzymes in the media or

inside the cells, rendering its derivatives less potent, since it is known that the half-life of colchicine in plasma is about 1 hour (Moffat, 1986). At concentrations of 0.1-1 µg/ml, colchicine can cause the mitotic arrest of dividing cells (both plant and animal cells) at metaphase by interfering with microtubule organization (Andreu et al, 1982). In astrocytes, colchicine (25 µM) can inhibit Rho kinase inhibitor Y-27632-induced astrocyte stellation (Abe et al, 2003).

The range of cytochalasin D concentration used in this study was based on other published experiments that used this drug. For example, cytochalasin D treatment (5 µM) reduces both spine and astrocyte motility (Haber et al, 2006). A concentration of 10 µM of cytochalasin D was sufficient to disturb the Ca2+ influx and inhibit calcium oscillations in astrocytes (Sergeeva et al, 1999). Golgi polarization was shown to be actin-independent in astrocytes using final concentrations of cytochalasin D of 0.5 and 2 µM, sufficient to affect the actin cytoskeleton (Magdalena et al, 2003). In our cell model, we found that a concentration of cytochalasin D over 2 µM disrupted the cell monolayers, rendering the scratch assay useless (Vincent-Héroux and Carbonetto, *unpublished*). That is why the highest concentration used was 1 µM. The astrocyte morphology does change from an extended flat cell to a rounded shape upon cytochalasin D treatment, as it was observed by many others (Ferrier et al, 1994; Sergeeva et al, 1999). Other compounds, such as the RGD peptide or function blocking antibody directed against α–DG or the alpha(1) integrin subunit, can inhibit process extension (Peng et al, 2008).

Dystroglycan and Rho GTPases

The formation of polarized protrusions in reactive astrocytes requires localized recruitment of Cdc42 to the cell edge and its rapid activation, which reaches a 4-fold increase 30 minutes after wounding. As a downstream effector of Cdc42, PKCζ activation reaches a maximum 1h after scratching and is sustained for at least 8h (Etienne-Manneville and Hall, 2001). Rac, on the other hand, is essential

for both the development and the maintenance of protrusions during migration but is not required for astrocyte MTOC polarization (Etienne-Manneville and Hall, 2001). Integrins control the translocation of GTP-bound Rac to lipid rafts at the front of the cell where it regulates cortical actin polymerization and membrane protrusion via its effectors, among which Pak plays an essential role (Grande-Garcia et al, 2005).

The results presented here support some of these findings but cannot be used to draw any conclusions. The Cdc42/Rac assay was monitoring molecular events that occur in the very first minutes after the initial stimulation. Several unsuccessful attempts, in which I could not detect activated Cdc42, led me to hypothesize that a lot of activated Cdc42 or Rac was lost and hydrolyzed to their GDP-bound state before certain steps of the protocol were completed. Moreover, protein detection from astrocyte monolayers has been a challenge, even for dystroglycan.

To verify if this issue was linked to the time of execution of the procedure, I modified certain parameters of the protocol, such as the centrifugation time to clear the lysate and the Bradford assay to measure the protein concentration of the different samples. From the initial protocol, I changed the centrifugation time to clear the cell lysate from 15 to 10 minutes. As part of the optimization process, I decided to use equal volumes of lysates rather than equal amounts of proteins throughout the experiment, and determine protein concentrations afterwards. The results, which are presented here, were that I could finally detect activated Cdc42 and Rac, with signal patterns similar to what has been reported in the literature. That confirmed the assay was working in our cell model, although it needed further optimization with respect of timing the different steps of the protocol.

In this assay, the differences between the protein amounts loaded, calculated afterwards, are not dramatic, but this was a weakness of this experiment. The protein concentrations should be measured before incubating the lysates with the

coupled GST fusion protein. Further optimization is needed in order to determine the protein concentrations without compromising activated Cdc42 detection. The assay would also gain more precision if quantitative densiometric analysis was used to measure normalized signal intensities.

The DGC protein complex was first suggested to be a signal transduction complex in 1992 (Madhavan et al, 1992). Their hypothesis was that in the sarcolemma, just as integrins bind fibronectin to initiate signaling, the DGC complex acts as a laminin receptor which initiates signaling upon laminin binding. Indeed, a growing body of evidence links components of the DGC, dystroglycan in particular, to Rho GTPases signaling.

The dystroglycan protein contains adaptor binding domains for several SH2 domain-containing signaling molecules. A DG:ezrin:Dbl complex was found to be targeted to the membrane by dystroglycan where it drives local Cdc42 activation and the formation of filopodia in fibroblasts (Batchelor et al, 2007). The same group found that depletion of dystroglycan inhibited Cdc42-induced filopodia formation. In addition, they were the first to show co-localization of Cdc42 and dystroglycan at the tips of dynamic filopodia.

Another group showed that in muscular atrophy, not only the proteins of the DGC complex were decreased, but activated Cdc42 was also decreased. Furthermore, Rac1 and Cdc42 were physically associated with the complex containing β–DG (Chockalingam et al, 2002). In complement with these findings, binding of laminin α1-chain LG4-5 domain to α–DG was shown to cause tyrosine phosphorylation of syntrophin and initiate Rac1 signaling in skeletal muscle (Zhou et al, 2006).

In our model, we hypothesized that Cdc42 activation would be perturbed in monolayers of DG null astrocytes stimulated by multiple scratching. I intended to perform a Cdc42/Rac activation assay with wild-type primary astrocytes and DG-null, wild-type and beta(1)-null integrin ES cell-derived astrocytes. These cell

lines have been successfully developed by our laboratory in the course of the project. A difficulty of the proposed experiment was to generate astrocytes from ES cells, a very delicate, one month process, and synchronize the four cell lines in order to perform the assay, since they had all a different growth rate, especially after the second passage. Optimization of the cell culture methods and the activated Cdc42 assay adapted to this particular model has been initiated and experimental success may lead to a better understanding of dystroglycan signaling in astrogliosis.

The *in vitro* wound assay

The *in vitro* scratch-induced wound assay is particularly suitable to study the regulation of cell-matrix interaction during migration. The desired matrix can be uniformly coated to the surface of a culture dish on which cells can be grown to confluence to form a homogenous monolayer. A major advantage of this assay is its compatibility with other methods such as microinjection or gene transfection. It can be easily adapted for fluorescence microscopy, including live cell imaging, allowing analysis of intracellular signaling during cell migration of individual cells at the leading edge of the scratch.

Among the disadvantages and limitations of the *in vitro* scratch assay, it is not well adapted for chemotaxis analysis as in the Boyden chamber assay, since no chemical gradient is established, neither on the matrix nor in the media. Time wise, the assay takes a relatively long time to perform, as nearly one week is needed to grow a sufficient amount of cells in a 175 cm^2 flask in order to plate them at high density in 24-well cell culture plate. Then, it takes two or three days for the formation of a confluent cell monolayer and several hours to monitor the closing of the scratch.

Primary astrocytes provide a good physiological model. Homogenous astrocyte cultures can be obtained in large quantities, which allow biochemical analysis

and they are resistant to frequent handling. In addition, the migration of astrocytes induced in this assay mimic the cell migration during wound healing *in vivo*. The present study support the pioneer work made by Etienne-Manneville *et al* in developing this method and confirms it is a powerful tool to investigate the molecular mechanisms controling cell adhesion, polarization and migration.

Conclusion and Summary

Dystroglycan and integrins are both thought to play a major role in the regulation of astrocyte behaviour. The issue of their respective contribution was not raised in this study. For example, in the basolateral membrane of intestinal epithelial cells, the DG complex was shown to co-precipitate with beta(1) integrin. In these cells, activation of DG receptors by laminin-1 enhanced the interaction between beta(1) integrin and laminin-1, whereas activation of DG receptors by laminin-2 reduced the interaction between beta(1) integrin and laminin-2, suggesting a possible interaction among these proteins (Driss et al, 2006). Thus, these receptors may have cooperating or opposing roles depending on the molecular setting.

Astrocytes are mainly responsible for the formation of the glial scar that occurs following a trauma to the CNS and ECM receptors may play and important role in this process. Dystroglycan and beta(1) integrins implication in wound healing has been reported in other cell models such as in airway epithelial cell repair (White et al, 1999; 2001) and regeneration of shearing-type muscle injury (Kääriäinen et al, 2000). While the formation of a glial scar is essential to restore the homeostasis of the CNS and the blood-brain barrier, the scar presents a mechanical and molecular impediment to the regeneration of axons and the re-establishment of normal function. Understanding the underlying cellular pathways of astrocyte wound healing will be important for developing new therapeutic targets to selectively alter specific molecular process in order to promote CNS regeneration.

The present study investigated the role of extracellular matrix receptors in astrocyte adhesion and migration. The cell distribution of DG was determined by immunofluorescence microscopy and compared with that of beta(1) integrins, using *in vitro* adhesion and wound healing assays. DG and integrins distribute differentially depending on the cell spreading stage, and both receptors get recruited to the cell leading edge in scratch-induced migration. We questioned the unresolved issue of DG association with GM1-rich signaling microdomains and proposed a model where DG is recruited to GM1-rich lipid rafts upon activation of specific migration signals. Using cytoskeleton disrupting agents, we concluded that the MT network was mainly responsible for protrusion elongation in the migrating astrocyte but this process is not actin-independent. In addition, cortical actin localization to the leading edge of the reactive cell is independent of the polarized microtubule network. Three ES cell-derived astrocyte cell lines were successfully developed: wild type, DG-null and beta(1) integrin-null in order to further the analysis of the role of DG in Rho GTPases signaling. We conclude that tightly coordinated signaling pathways dictate the cellular distribution of ECM receptors in non-stimulated and reactive astrocytes. Information obtained from the simple scratch wound system will eventually need to be tested *in vivo* using a stab wound model of scar formation in the brain of rats or mice.

Figures

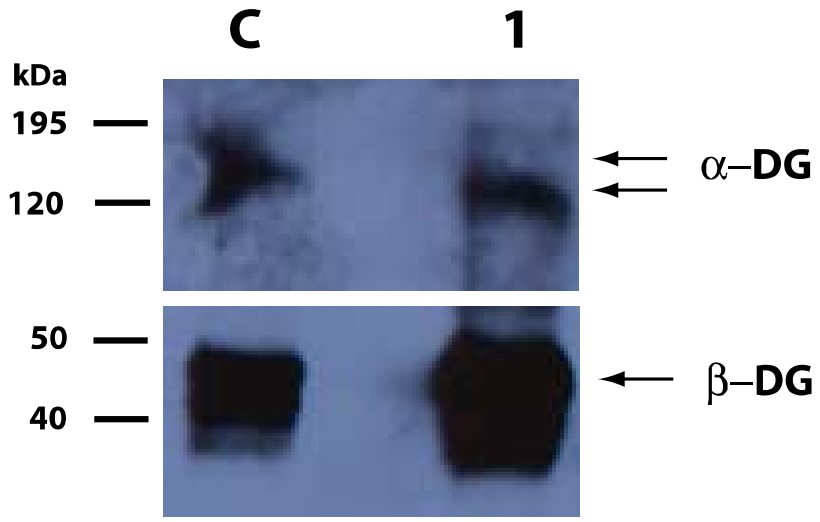


Figure 1: Dystroglycan expression in type 1 primary rat astrocytes.
Cultures of primary astrocytes were fractionated as described in materials and methods and fractions were electrophoresed in a 8% (top panel) and 12 % (lower panel) SDS polyacrylamide gel and transferred on to nitrocellulose membranes. Blots were probed with antibody to α-DG (antibody IIH6 (1:800), upper panel) and antibody to β-DG (1:5000; lower panel; the doublet for astrocyte β-DG may represent degradation products). Reactivity was revealed using appropriate HRP-labeled secondary antibodies. Lane C: Skeletal muscle microsomial extract as positive control (5 µg). Lane 1: Astrocyte microsomal fraction (7 µg).

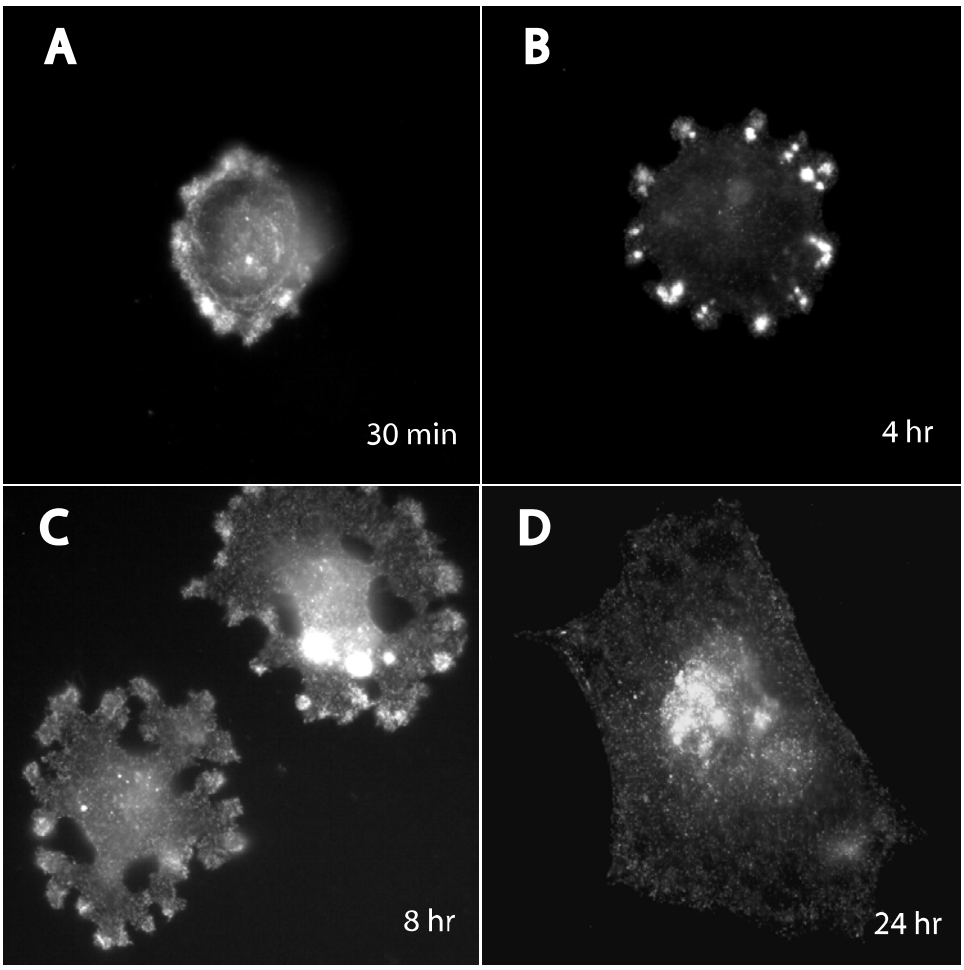


Figure 2: Distribution of β–DG over the 4 spreading stages of primary astrocytes. Primary cultures of rat astrocytes were treated with trypsin and the detached cells were replated at low density (2,5 x 10^5 cells/ml) on glass coverslips, pre-coated with laminin (100 µg/ml), in 24-well culture plates. The cells were allowed to spread for different time points: (A) 30 min, (B) 4h, (C) 8h and (D) 24h at 37 ^oC in a 5% CO_2 atmosphere. Adherent astrocytes were then fixed with 4% PFA and permeabilized with 0.25% Triton X-100/PBS for 10 min. β–DG was visualized by immunofluorescence microscopy.

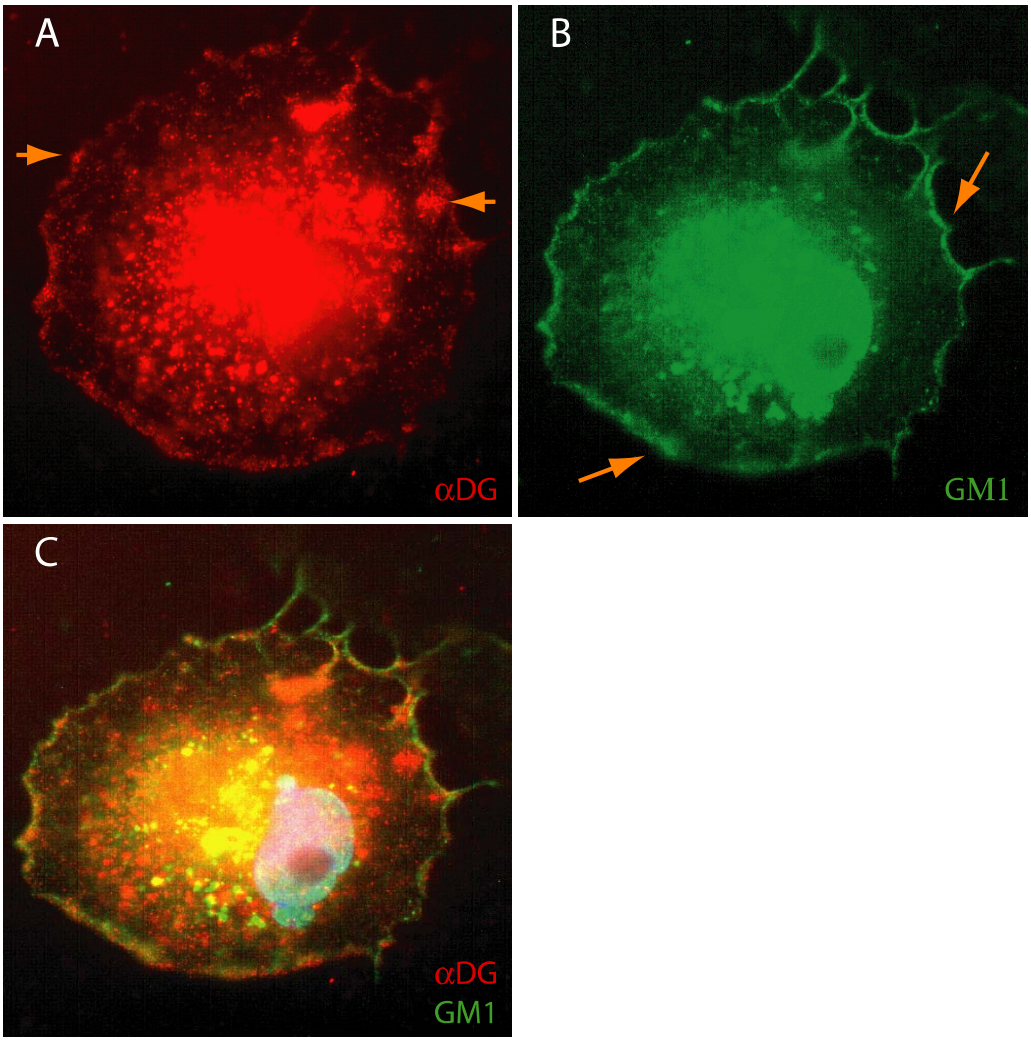


Figure 3 : Distribution of α–DG and the lipid raft marker GM1 in adherent primary astrocytes. Primary rat astrocyte culture were trypsin-detached and allowed to adhere at low cell density to laminin-coated glass coverslips in 24-well culture plates for 1 hr at 37°C, 5% CO_2. Adherent cells were then fixed with 4% PFA and permeabilized with 0.25% Triton X-100/PBS for 10 min. α-DG (red) and GM1 (green) were stained with the IIH6 (1:200) antibody and FITC-coupled cholera toxin B subunit (1:200), respectively. (A) α-DG; (B) GM1; (C) merge. Short arrows: DG clusters. Long arrows: GM1 clusters.

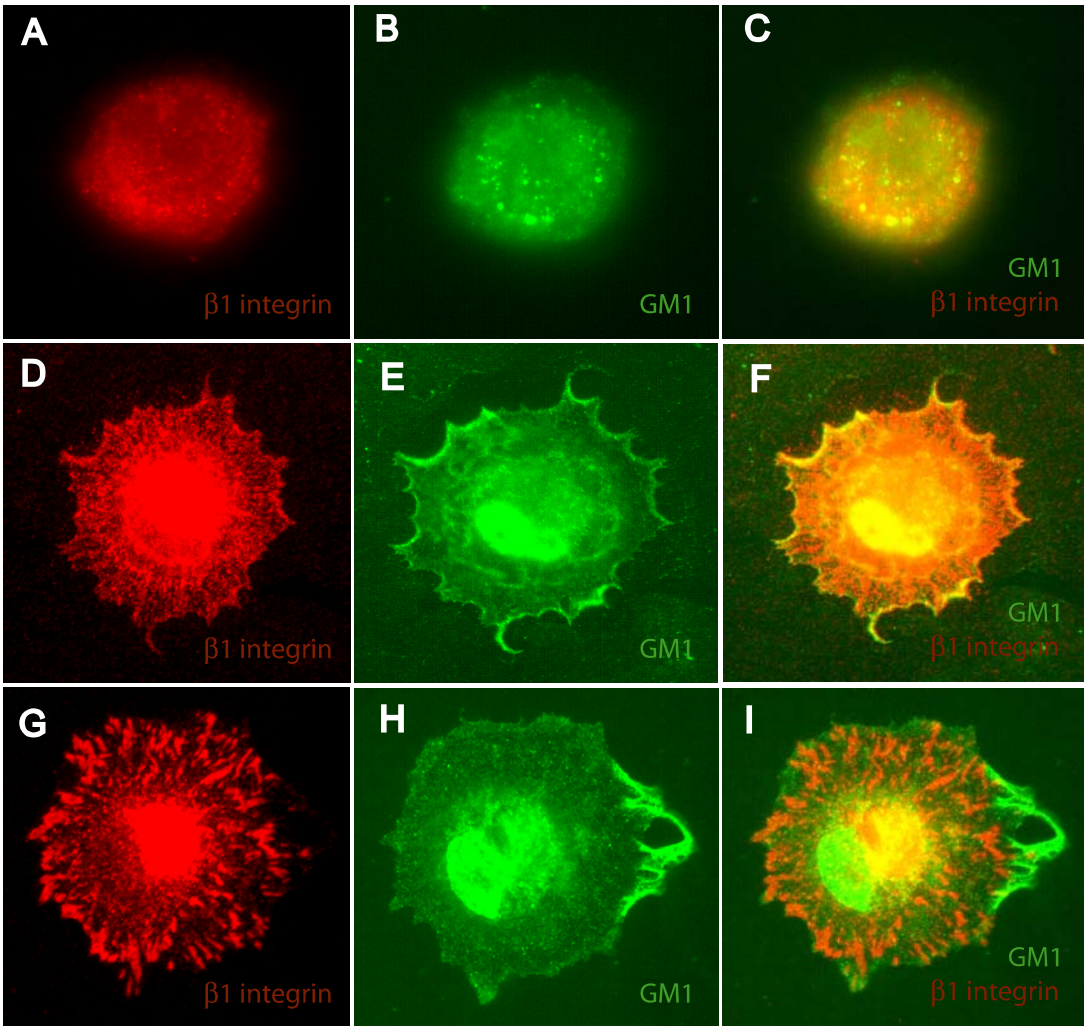


Figure 4: Distribution of beta(1) integrin with lipid raft marker GM1 in early stages of astrocyte adhesion. Primary cultures of rat astrocytes were treated with trypsin and the detached cells were replated at low density (2,5 x 10^5 cells/ml) on glass coverslips, pre-coated with fibronectin**,** in 24-well culture plates. The cells were allowed to spread for 1 hr at 37 ^oC in a 5% CO_2 atmosphere. Adherent astrocytes were then fixed with 4% PFA, permeabilized, and β1 integrin (red) and GM1 (green) were stained with the polyclonal β1 integrin (1:200) antibody and FITC-coupled cholera toxin B subunit (1:200), respectively. Panel (A-D-G) β1 integrin; (B-E-H) GM1; (C-F-I) merges. Panel (A-B-C) Cell spreading stage 1; (D-E-F) Cell spreading stage 2; (G-H-I) More advanced state of stage 2.

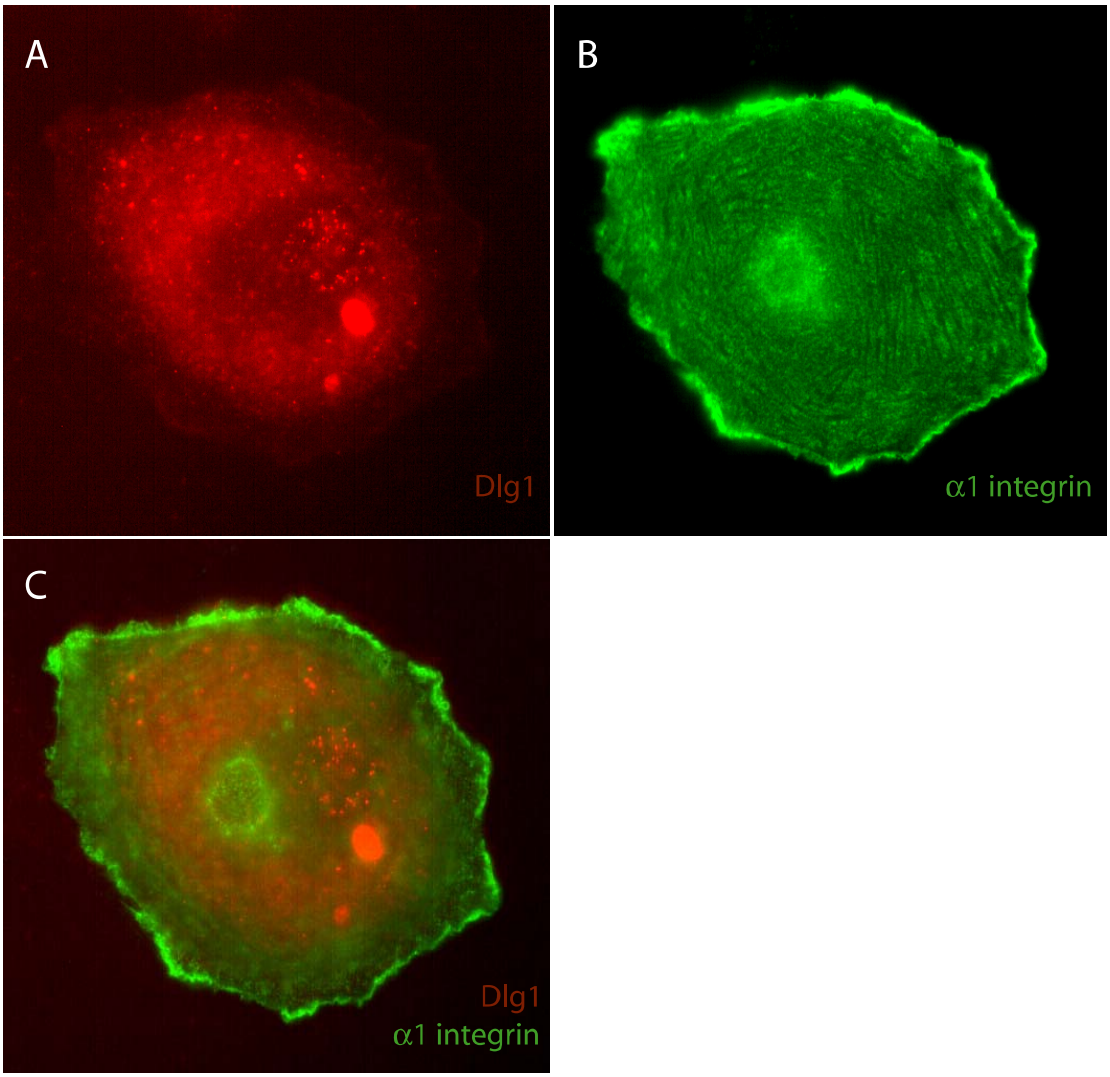


Figure 5: Distribution of the alpha(1) integrin subunit and Dlg1 in adherent astrocytes. Primary rat astrocyte culture were trypsin-detached and allowed to adhere at low cell density to laminin-coated glass coverslips in 24-well culture plates for 1 hr at 37°C, 5% CO_2. Adherent cells were then fixed with 4% PFA and permeabilized with 0.25% Triton X-100/PBS for 10 min. Dlg1 (red) and the alpha(1) integrin subunit (green) were stained with the antibody against Dlg1(1:75) and the 3A3 antibody (1:250), specific to alpha(1) integrin. (A) Dlg1; (B) α1 integrin; (C) merge.

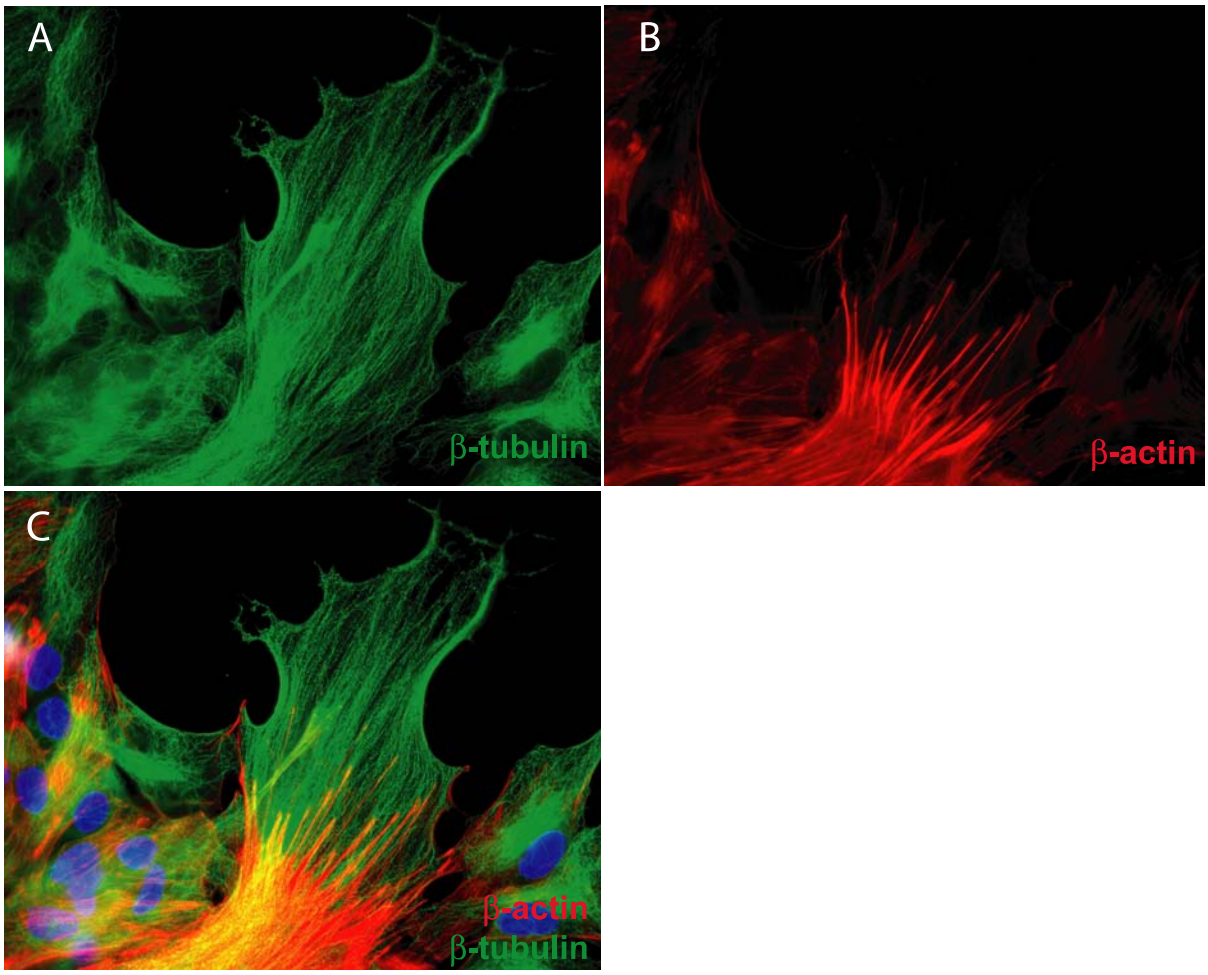
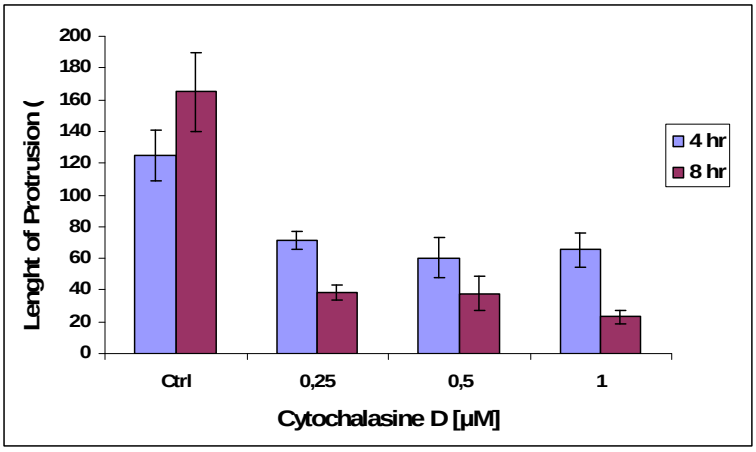


Figure 6: Microtubule-rich protrusions in reactive astrocytes.

Subcellular organization of the actin (red) and microtubule (green) cytoskeletons during scratch-induced protrusion formation was determined by immunofluoresence microscopy. Confluent monolayer cultures of primary rat astrocytes were scratched with a 18G blunted needle and incubated 8 hr at 37^{o}C, 5 % CO_2. Cells were then fixed, permeabilized and stained for β-actin with TRITC-conjugated phalloïdin (1:5000) and a specific antibody against β-tubulin (1:250). Panel (A) β-tubulin; (B) β-actin; (C) merge.

A



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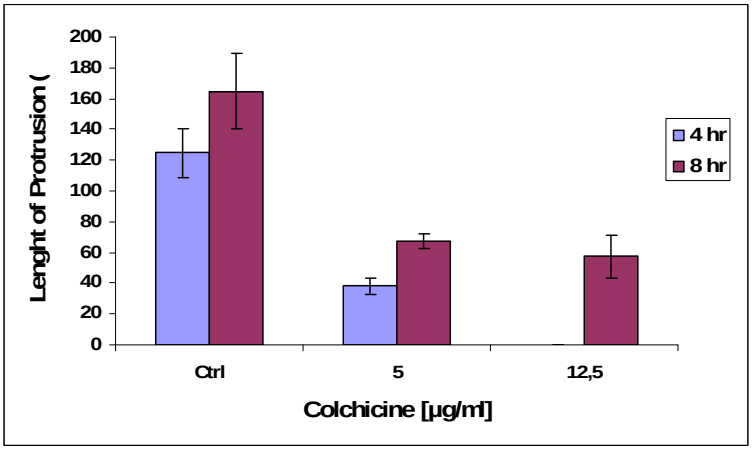

Figure 7: Effect of cytochalasin D and colchicine on process extension in reactive astrocytes. Confluent monolayer cultures of primary rat astrocytes grown on laminin were treated 0,25, 5 or 1 µM of cytochalasin D, or with 5 or 12,5 µg/ml of colchicine.. Astrocytes were immediately wounded by scraping across the monolayer with a blunted 18G needle and incubated 4hr and 8 hr at 37°C, 5% CO_2. Cells were then fixed and stained with Coomassie blue. Photomicrographs were taken at 200x magnification. For each conditions, the mean length of protrusion was calculated from measurements of 100 cells at the leading edge of the scratch, measured from the top of the nuclei to the tip of the protrusion perpendicularly to the scratch, using Northern Eclipse Version 7.0 image analysis software. (A) Effect of cytochalasin D; (B) Effect of colchicine.

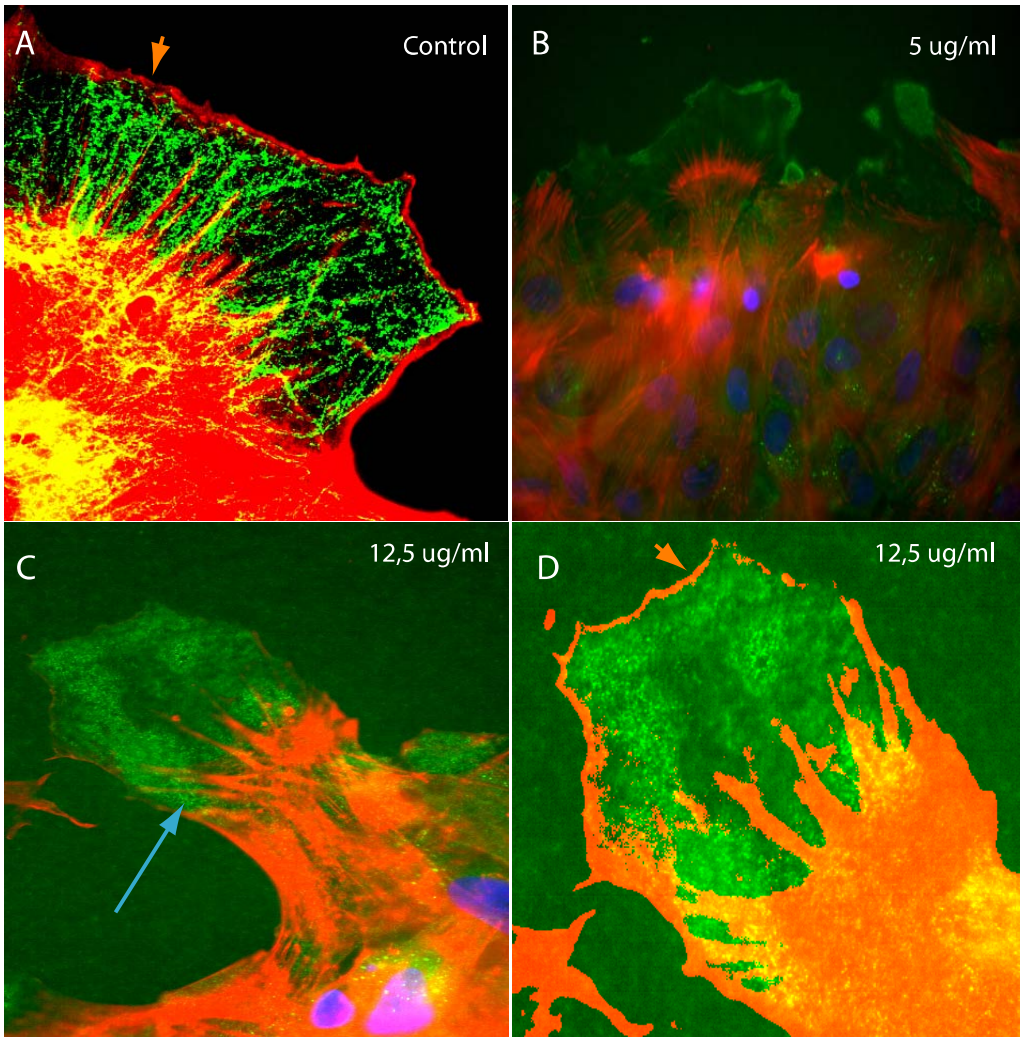

Figure 8: Visualization of cortical actin after colchicine treatment in wounded astrocytes. Subcellular organization of the actin (red) and microtubule (green) cytoskeletons during scratch-induced protrusion formation was determined by immunofluoresence microscopy. Confluent monolayer cultures of primary rat astrocytes were treated with colchicine (5 or 12,5 µg/ml)scratched with a 18G blunted needle and incubated 8 hr at 37^{o}C, 5 % CO_2 . Cells were then fixed, permeabilized and stained for β-actin with TRITC-conjugated phalloïdin and a specific antibody against β-tubulin. All panels are merge images. Magnifications: Panel (A) Untreated control, 630x; (B) 5 g/ml colchicine treatment, 200x; (C) 12,5 g/ml colchicine treatment, 630x; (D) Magnification of Fig.8C, 1000x. Radial actin bundles: blue arrow; Cortical actin: orange arrows.

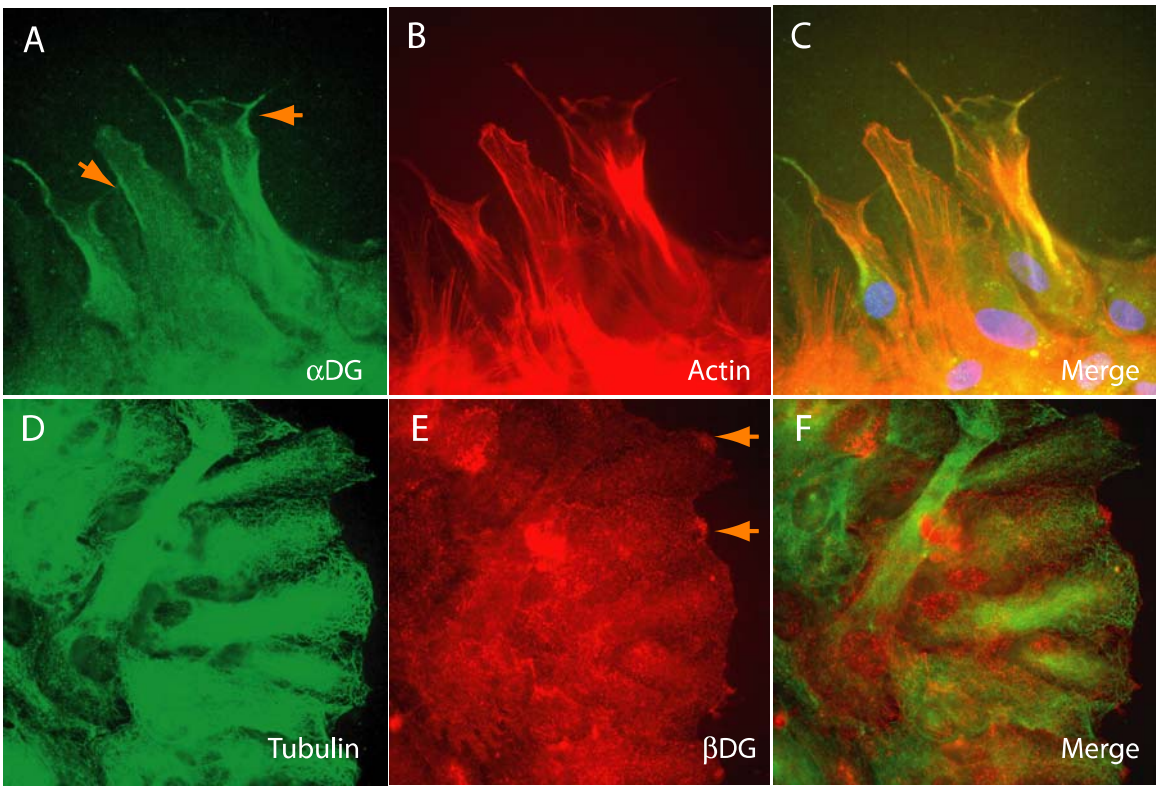

Figure 9 : Subcellular localization of the DG subunits and the cytoskeleton in reactive astrocytes. The subcellular distribution of DG subunits and the actin and MT cytoskeletons during scratch-induced protrusion formation was determined by immunofluoresence microscopy. Confluent monolayer cultures of primary rat astrocytes grown on laminin were scratched with a 18G blunted needle and incubated 8 hr at 37°C, 5 % CO_2. Cells were then fixed, permeabilized and stained for α-DG, β-DG, β-actin and β-tubulin with the VIA4.1 antibody, β-DG antibody, TRITC-conjugated phalloïdin and β-tubulin antibody, respectively. Panel (A-C) α–DG (green) and β-actin (red), merge; (D-F) β-DG (red), β-tubulin (green), merge.

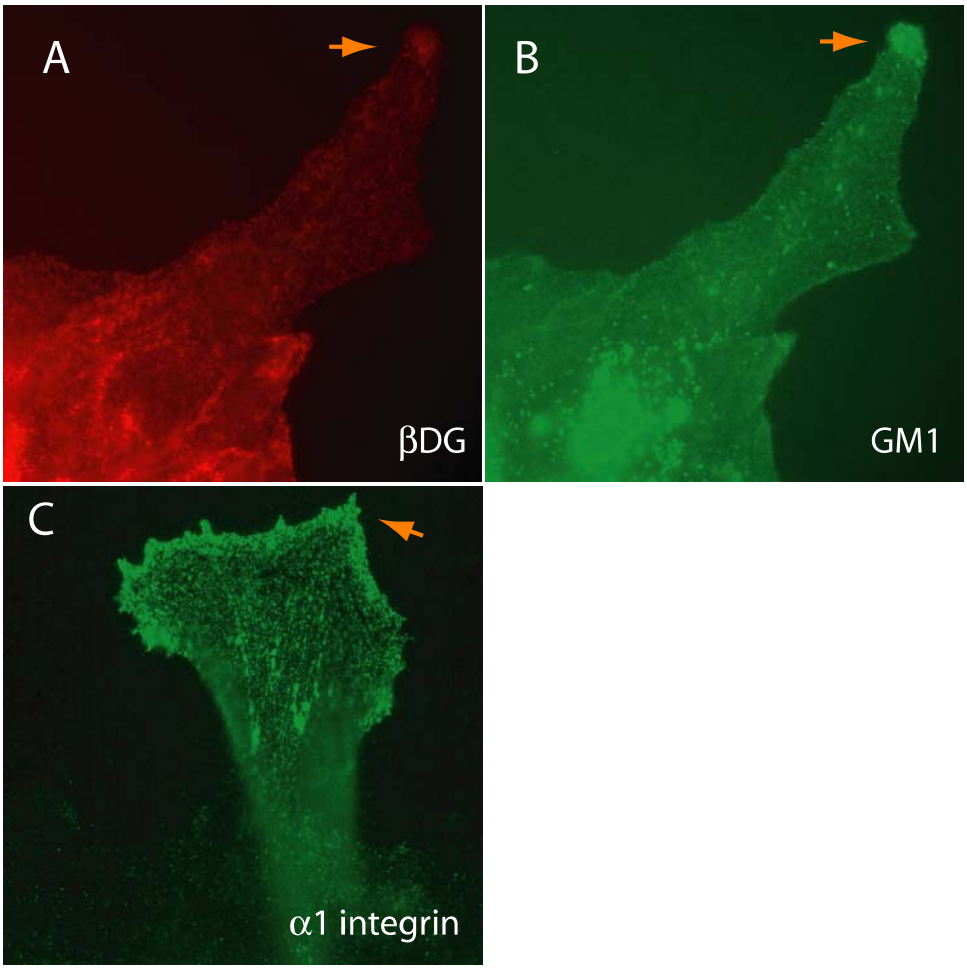

Figure 10: Cellular localization of alpha(1) integrin, β–DG and GM1 in astrocytes during scratch-induced process extension. The cellular localization of alpha(1) integrin, β-DG and GM1 during scratch-induced protrusion formation was determined by immunofluoresence microscopy. Confluent monolayer cultures of primary rat astrocytes grown on laminin were scratched with a 18G blunted needle and incubated 8 hr at 37^{o}C, 5 % CO_2. Cells were then fixed, permeabilized and stained for β-DG, the lipid raft marker GM1 or the α1 integrin, with the β-DG antibody, FITC-coupled cholera toxin B and antibody 3A3, respectively. Panel (A) β-DG (red); (B) GM1 (green); (C) α1 integrin (green). Panel (A) and (B) represent the same cell while panel (C) represents a different cell. Arrows point to the localization of the molecules at the tip of the extending process.

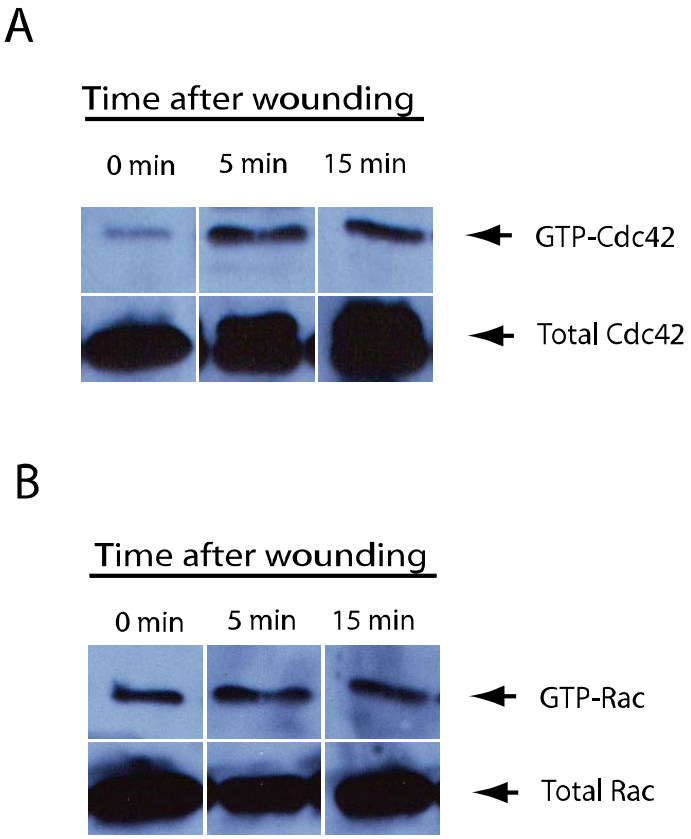

Figure 11: Activation of Rho GTPases in wounded astrocytes.

Confluent monolayer cultures of primary astrocytes were multi-scratched and activated Cdc42/Rac was detected 5 and 10 minutes after wounding using a Cdc42/Rac assay. Lysates from wounded astrocytes, incubated 60 min with coupled GST or GST-PAK-CRIB beads, and total lysates were electrophoresed on a 12% SDS-polyacrylamide gel and transferred on to a nitrocellulose membrane. Total and GTP-bound Cdc42 was visualized on Western Blot. The same membrane was stripped and blot for total and GTP-bound Rac1.

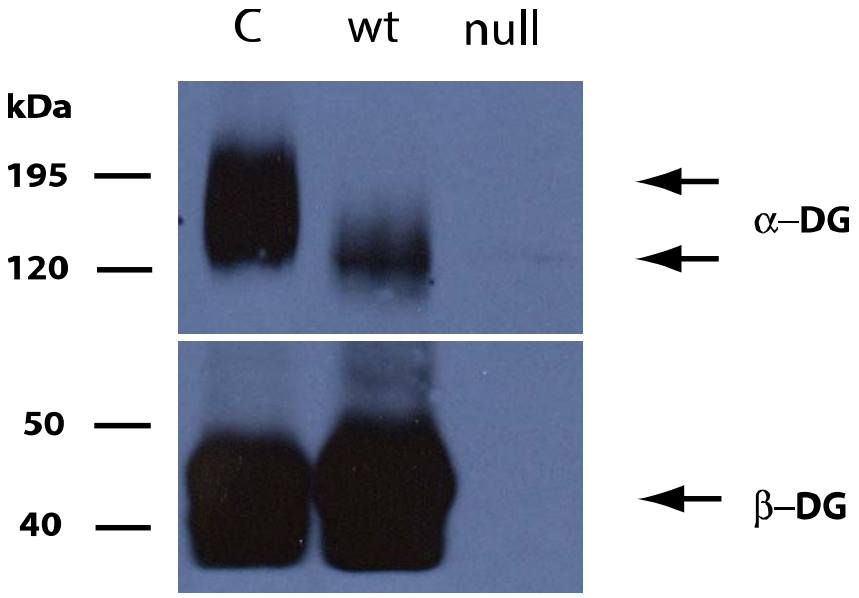

Figure 12: DG expression in ES cell-derived astrocytes.

Cultures of ES cell derived astrocytes were fractionated as described in materials and methods and microsomial fractions (10 µg) of wild-type and DG-null cells were electrophoresed in a 8% (upper panel) and 12 % (lower panel) SDS polyacrylamide gel and transferred to nitrocellulose membranes. Blots were probed with antibody to α-DG (antibody IIH6 (1:800), upper panel) and antibody to β-DG (1:5000; lower panel). The smeared signals may represent degradation products. Reactivity was revealed using appropriate HRP-labeled secondary antibodies. Lane C: Skeletal muscle microsomial extract as positive control (5 µg).

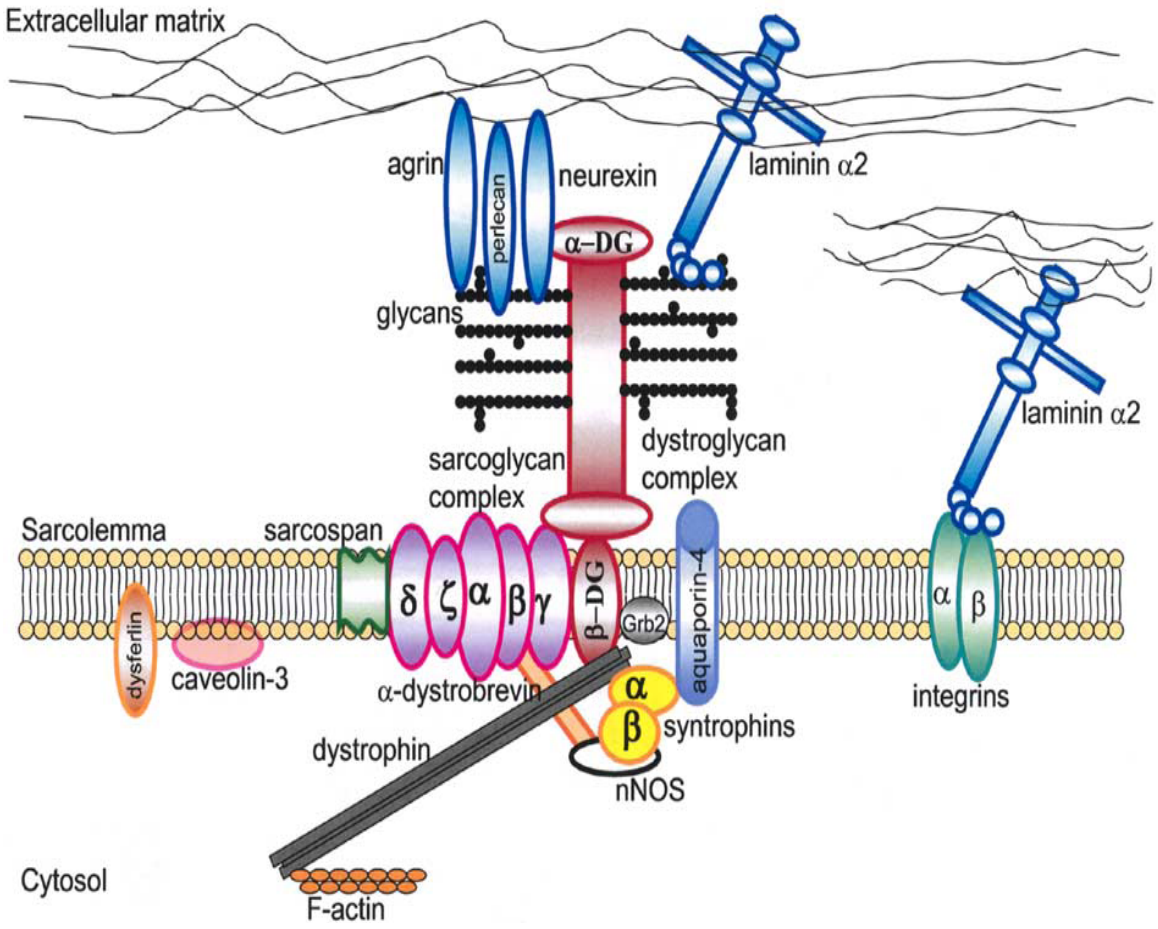

Figure 13: The dystrophin glycoprotein complex and other membrane associated proteins (adapted from Cohn, 2005).

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Appendix I – Animal use Protocol

		DATE
COs		
DB		
APPROVED		

www.mcgill.ca/research/compliance/animal/forms/

For Office Use Only:
Protocol #: 4720
Approval end date: MARCH 31, 2007
Facility Committee: MGH
Renewal#: 1st 2nd

McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator: Dr. Salvatore Carbonetto — Protocol # 4720

Protocol Title: Control of cell death by dystrophin associated proteins — Phone: (514) 934-1934 Ext. 44237

Unit, Dept. & Address: Neurology,/1650 Cedar Avenue, Montreal, Quebec H3G 1A4 — Fax: (514) 934-8261

Email: sal.carbonetto@mcgill.ca — Level: C — Funding source: CIHR 6477

Start of Funding: April 01 2005 — End of Funding: March 31 2009

Emergency contact #1 + work AND home phone #s: S. Carbonetto 934-1934 x 44237, (514) 932-7016

Emergency contact #2 + work AND home phone #s: Waris Ali Shah, 934-1934 x 44575, (450) 550-9275

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information	Occupational Health Program *	Signature *"Has read the original full protocol"*
Salvatore Carbonetto	Principal Investigator	Theoretical Training	Yes	
Waris Ali Shah	Postdoctoral Fellow	Theoretical/Workshop 2005	Yes	
Nadia Melian	Graduate Student	Theoretical/Workshop 2005	Yes	
Mathieu Tremblay	Graduate Student	Animal Workshop 2005	Yes	
Huashan Peng	Postdoctoral Fellow	Theoretical/Workshop 2005	Yes	
jonathan Vincent-Heroux	Graduate Student	Theoretical/Workshop 2005	Yes	

* Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.

Approved by: FACC approved - no comm. - lp.

2. Approval Signatures

Principal Investigator/ Course Director		Date: 25 May 2006
Chair, Facility Animal Care Committee	Kathryn Gill	Date: June 6/06
UACC Veterinarian		Date: June 6, 2006
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		Date:
Approved Animal Use Period	Start: April 1, 2006	End: MARCH 31, 2007

3. Summary (in language that will be understood by members of the general public)

AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).*

In skeletal muscle dystroglycans (DGs) form the core of a large complex of dystrophin associated proteins which

JUIL. 2005