Integrin function and regulation in development.

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Integrin function and regulation in development

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ABSTRACT Integrins are a large family of membrane receptors, consisting of α and β subunits, that play a pivotal role in the interaction of cells with the extracellular matrix. Such interaction regulates the organization of cells in organs and tissues during development as well as cell differentiation and proliferation. We have shown that unfertilized oocytes express integrins that might be important during fertilization. We also analyzed nervous system and muscle tissue development showing that integrin expression is precisely regulated during organization of these tissues. The results indicate that two distinct integrin α subunits mediate the outgrowth of processes in nerve and glial cells. α1 integrin, a laminin receptor, is up-regulated by nerve growth factor and other differentiation stimuli and is involved in neurite extension by nerve cells. In contrast, process extension by glial cells is likely to involve the αV integrin. Moreover, the latter integrin subunit is also transiently expressed in muscle of the embryo body where it localizes predominantly at developing myotendinous junctions. After birth this integrin disappears and is substituted by the α7 subunit. At the same time, important changes also occur in the expression of the associated β subunit. In fact, the β1A isoform which is expressed in fetal muscles, is substituted by β1D. These isoforms are generated by alternative splicing and differ in only a few amino acid residues at the COOH terminus of the protein. This region of the molecule is exposed at the cytoplasmic face of the plasma membrane and is connected to the actin filaments. Our results show that β1D, which is expressed only in striated muscle tissues, binds to both cytoskeletal and extracellular matrix proteins with an affinity higher than β1A. Thus, β1D provides a stronger link between the cytoskeleton and extracellular matrix necessary to support mechanical tension during muscle contraction. These results indicate that cells can regulate their interactions with the extracellular matrix by changing their expression of α integrin subunits and thus ligand specificity, or by more subtle changes involving alternative usage of different cytoplasmic domains. The important role of both α and β integrin subunit cytoplasmic domains during development is further illustrated by the analysis of targeted mutations which we have generated by homologous recombination in mice..

KEY WORDS: *integrin, oocyte, muscle, nerve, glia, targeted mutations.*

Introduction

Integrins are hetrodimeric membrane receptors that regulate the interactions of cells with the extracellular matrix. The analysis of these molecules started with the discovery of the fibronectin receptor as a cell surface glycoprotein of 110-130 kDa resistant to trypsin cleavage (Tarone *et al.*, 1982; Giancotti *et al.*, 1985) and capable to bind the Arg-Gly-Asp sequence in fibronectin (Pytela *et al.*, 1985). Soon it became clear that the structural features of the fibronectin receptor were similar to those of several other cell-cell and cell-matrix adhesion receptors that were collectively indicated as integrins to underline their ability to functionally integrate the

Abbreviations used in this paper: ANP, Atrial Natriuretic Peptide; JNK, Jun N-terminal Kinase; MAPK, Mitogen Activated Protein Kinase; NGF, Nerve Growth Factor; PCR, Polymerase Chain Reaction; RA, Retinoic Acid; INS, Insulin; PMA, Phorbol Myristate Acetate.

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extracellular matrix with the cytoskeleton across the plasma membrane (Hynes, 1987).

Integrins consists of two transmembrane subunits α and β that form a non covalent heterodimer. Both subunits contribute to the formation of the ligand binding pocket, but the α subunit is likely to play an important role in determining the binding selectivity since often heterodimers sharing a common β subunit, but with distinct α chain, bind different ligands. The ligand binding requires the presence of divalent cations, and Ca^{++}, Mn^{++} and Mg^{++} can differentially contribute to the binding affinity. Indeed the binding affinity state of many integrins can be regulated at the cell surface by several stimuli allowing to regulate the intensity of adhesion during cellular interactions and migration (Hynes, 1992).

At the cytoplasmic face of the plasma membrane integrins bind to cytoskeletal proteins of the actin contractile system. This can be clearly appreciated in stationary cell in culture where integrins are concentrated in small patches at the ventral cell surface in contact with the adhesive substratum known as "focal adhesions". At these sites are also concentrated cytoskeletal proteins such as vinculin, talin, α-actinin, paxillin and tensin that are bridging integrins to the actin filaments.

Beside their ability to organize the cytoskeleton, integrins can function as positional receptors generating intracellular signals that control cell survival and proliferation (Hynes, 1992; Giancotti, 1997). Cell matrix adhesion *via* integrins protects from apoptosis allowing cell survival (Frisch and Ruoslahti, 1997). This represents an important control mechanism in tissue formation and homeostasis to eliminate cells that have not reached, or lost, their proper location within the tissue. Moreover, interaction with the extracellular matrix is required for proliferative response to mitogens. The anchorage-dependent cell growth is a well known property of most cell types. It is now clear that integrins generate consensus signals necessary to obtain a full mitogenic response upon growth factor stimulation (Giancotti, 1997).

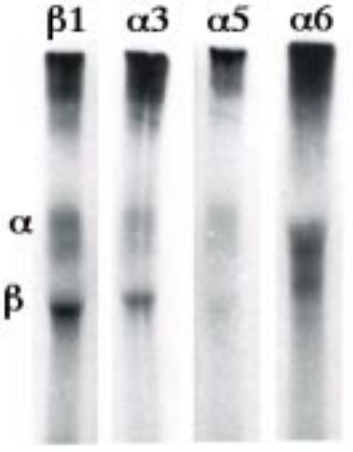

Fig. 1. Unfertilized mouse oocytes express three integrin complexes at their surface. *Membrane proteins exposed at the cell surface of unfertilized mouse oocytes were labeled using the lactoperoxidase catalyzed iodination procedure. After detergent extractions, labeled proteins were immunoprecipitated with integrin-specific antibodies and visualized after SDS-PAGE and autoradiography. The positions of the α and β subunits are indicated on the side and the subunit specific antibodies used are indicated on the top of each lane.*

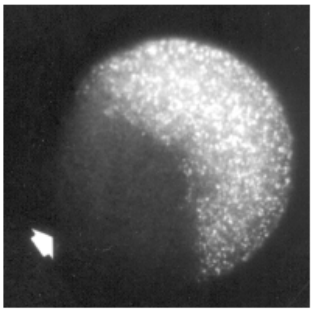

Fig. 2. Integrin α6 is expressed on the microvillous area of the unfertilized mouse oocyte. *Oocytes were labeled with antibodies specific for integrin α6 followed by fluorescein-labeled secondary antibodies. The white arrow points to the smooth region of the oocyte surface where the α6 integrin is absent.*

Thanks to their ability to mediate cellular interactions, motility and signaling, integrins play a crucial role in tissue and organ morphogenesis during development. One of the first example of the role of integrins in development came from the work of J.P. Thiery and coworkers who showed how neural crest cells utilize integrins to migrate along the fibronectin rich matrix around somites to reach their final destination in the developing embryo (Duband *et al.*, 1990). The role of integrins in development has been further demonstrated by the genetic approach. Both naturally occurring or engineered mutations in *Drosophila* and mouse have proved the role of different integrin subunits at various stages of embryonic development (Fassler *et al.*, 1996).

Integrins are expressed at very early stages of development

Information on the spatio-temporal expression of integrins are crucial to investigate their role in development. For this reason we and others have investigated this aspect in some details. This investigation was initially hampered by the lack of specific reagent useful to identify integrin subunits in mouse or chicken. When antibodies capable to react with integrins from all animal species become available this analysis became feasible. We concentrated our analysis on the very early stage and showed that several integrin heterodimers are already expressed at the surface of unfertilized mouse oocyte (Tarone *et al.*, 1993). Using both reverse transcription PCR and immunoprecipitation with specific antibodies, we showed that mouse oocytes express at their surface α3β1, α5β1 and α6β1 heterodimers (Fig. 1). These integrins can function as receptors for laminins and fibronectin. Other β1 and β3 containing integrins, such as α1β1, α2β1, α4β1 and αVβ3, are not present at oocytes surface, thus, showing a specific pattern of expression. Surface expression of α6β1 integrin was also demonstrated by immunofluorescence and immunoelectron-microscopy. This integrin is mainly confined to the microvillous area of the oocyte surface where sperm-egg interaction and fusion occurs (Fig. 2). This suggested a possible role of α6β1 in fertilization process. Indeed recent work supported the notion that oocyte integrins, and α6β1 in particular, are involved in oocyte-sperm binding and fusion. Sperm express at their surface molecules of the ADAM family that have structural motives capable to interact with integrins (the disintegrin motif). Sperm from mice lacking ADAM2 were shown to be deficient in sperm-egg membrane adhesion and sperm-egg fusion (Cho *et al.*, 1998). ADAM2, moreover, was found to bind specifically with α6β1 (Chen *et al.*,1999).

Oocyte integrins might also be important for cell-matrix interaction during the early phases of embryonic development. Thrombospondin is one of the matrix proteins found on unfertilized oocytes and on two cell stage embryos (O'Shea *et al.*, 1990).

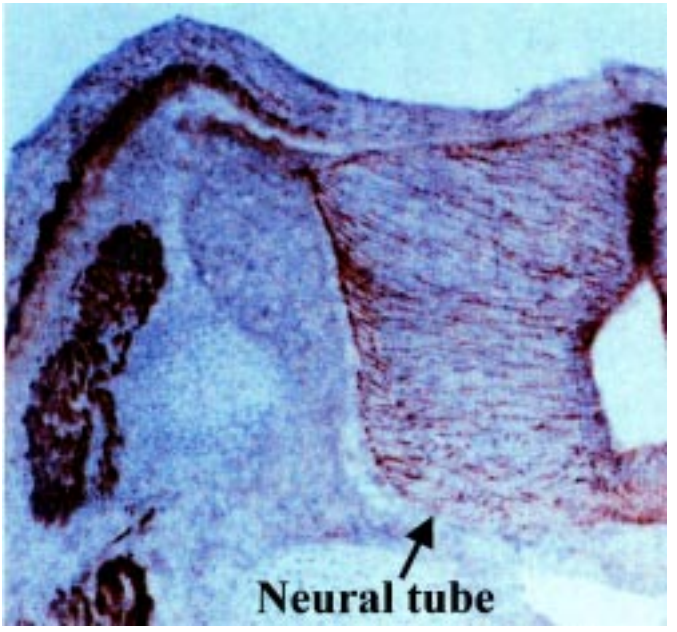

Fig. 3. Integrin αV is expressed in radial glial cells during mouse brain development. *Transverse section of a mouse embryo at E14.5, stained with αV antibodies followed by peroxidase labeled secondary antibodies. A region corresponding to the neural tube in the caudal region of the embryo is indicated by the arrow. m indicates the myotome.*

Thrombospondin contains an Arg-Gly-Asp sequence and promotes trophoblast spreading *in vitro* more efficiently than other matrix proteins suggesting that it might have a role in embryo implantation in the uterine wall. In addition to thrombospondin, laminin 1 is expressed at four cells stage (Dziadek and Timpl, 1985), while fibronectin and collagen type IV are first detected in the inner cell mass of the blastocyst (Wartiovaara *et al.*, 1979, Leivo *et al.*, 1980). The crucial role of β1 integrins in the early phases of the development has been lately directly demonstrated by the lethal phenotype of the β1 null mice. When expression of the β1 integrin subunit is abrogated by gene knock out, the embryo develops until day 5.5 at the blastula stage and fails at the stage of implantation in the uterine wall (Fassler and Meyer, 1995). The ability to develop until day 5.5 is not surprising since a small amount of β1 integrin can still be present in the homozygote embryo due to contribution from the maternal oocyte. The reason of failure at the stage of implantation are still not certain. Apoptotic death of inner cell mass cells has been detected in mutant embryos (Stephens *et al.*, 1995) suggesting that these cells fail to organize a proper extracellular matrix and do not get survival signals.

Integrin expression in neuronal differentiation

While β1 integrins are ubiquitously expressed, α subunits are present in specific tissues and their expression is likely to be regulated by morphogens. One of the tissues where cellular interactions play a pivotal role is the nervous system. Nerve cells undergo complex cycles of growth and migration during the development of the brain cortex in the embryo and cerebellar cortex in newborn mice. During the histogenesis of the central nervous system, neuroblasts are generated in the ventricular zone and migrate radially to generate the neuronal layers present in the brain cortex and cerebellum. The migration of neuroblasts is dependent on radial glial cells whose processes span the entire thickness of the neural tube wall extending from the luminal to the pial surface. Fascicles of radial glial cell provide a permissive substrate for neuroblast migration and axon guidance. Interestingly neuronal migration in brain cortex occurs around day 14.5 of embryonic life but occurs only postnatally in the cerebellum. Using specific antibodies, we have shown that αV integrin subunit is expressed in radial glial cells when neuronal migration occurs and it is down regulated when this process had ceased (Fig. 3) (Hirsch *et al.*, 1994). Expression of this integrin correlates, thus, with the guidance function of these glial cells.

Using antibodies capable to interfere with αV integrin-ligand binding on neuron-glial cell cultures from mouse embryo brain, Anton *et al.*, (1999) have recently shown that indeed αV integrin are required for optimal level of cell adhesion during migration of cortical neurons on glial cells. In addition neuronal cells utilize α3β1 integrin in this process as proved by the altered organization of cortical neuron layers in mice lacking this integrin subunit (Anton *et al.*, 1999).

While αV and α3 integrins have a crucial role in development of cortical neurons in the central nervous system other integrins seem to play a role in peripheral neurons. Using *in vitro* model systems consisting of PC12 pheochromocytoma cells and SY5Y and IMR32 human neuroblastomas, we have shown that the laminin receptor α1β1 is important in neuronal differentiation and neurite elongation (Rossino *et al.*, 1990; 1991). These cell lines grow *in vitro* as undifferentiated cells. However, upon exposure to Nerve Growth Factor (NGF) or retinoic acid (RA) for several hours, they stop proliferating and acquire a differentiated phenotype (Fig. 4). After such treatment PC12 and SY5Y or IRM32 cells become capable to elongate neurites on laminin, a matrix protein present in the nerve basal lamina *in vivo*. Treatment with NGF and RA induces 5-10 times increase in surface expression of α1β1 integrins, but leaves unaffected the levels of other integrin subunits (Fig. 5) (Rossino *et*

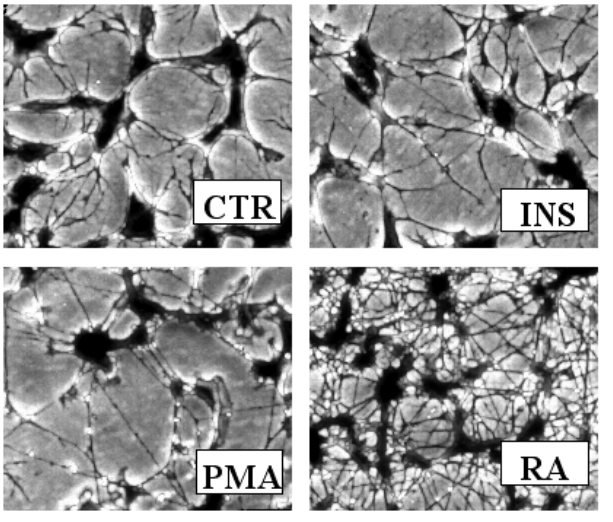

Fig. 4. Retinoic acid induces morphological differentiation of neuroblastoma cells *in vitro*. *SY5Y human neuroblastoma cells were grown in standard conditions (CTR) or treated for seven days with 1 μg/ml of insulin (INS), $1.6x10^{-8}$ M phorbol myristate acetate PMA (PMA), or $1x10^{-5}$ M retinoic acid (RA). Note the formation of a dense network of neurites induced by RA treatment.*

al., 1990; 1991). α1β1 act as cell surface receptor for laminin in these cells, as demonstrated by affinity chromatography experiments (Rossino *et al.*, 1990; 1991), and, thus, its up regulation by differentiation factors is likely to be a crucial event in determining the guidance of axons during nerve cell development.

Integrins in muscle development

Analysis of αV integrin expression with specific antibodies indicated a peculiar expression of this molecule during muscle development (Hirsch *et al.*, 1994). αV integrin is expressed very early during muscle development in the myotome of 10.5 days mouse embryos. At this stage of development both sclerotome and dermatome, that represent the two other cellular compartment of the somite, do not express αV integrin. At 12.5 days of development the αV appears to be expressed by primary myotubes in premuscle masses located between the vertebrae and in the body wall. In these muscles αV appears to be concentrated at myotubes ends. This distribution becomes even stronger at later stages (E15.5) where αV is highly concentrated at the apical extremities of myotubes clearly marking their spindle shape. This localization was clearly appreciated at muscle edges in proximity of the developing bones strongly suggesting a role of αV integrin in the organization of the early myotendinous junction (Fig. 6). At these sites, in fact, αV co-distributed with desmin, a muscle specific intermediate filament protein known to be concentrated at myotendinous junctions (Tidball, 1992). A localization of αV integrin at myotube ends was also demonstrated *in vitro* cultures of myogenic cells (Hirsch *et al.*, 1994). Myotendinous junctions do not form *in vitro* and thus it is likely that the subcellular localization of αV at two opposing ends of the elongated myotubes is driven by some intracellular mechanisms, rather than from clustering by matrix proteins from the extracellular site.

αV expression in skeletal muscle declined after E15.5 and the protein disappeared from adult mice muscles. Another integrin α subunit, α7, is instead present in adult myotendinous junctions where it is associated with β1 subunits and contributes to the mechanical stability of this junction during muscle contraction (Belkin *et al.*, 1996) (Fig. 7). Since α7 integrin is also expressed in muscles at early stages of development (E10.5), the selective presence of αV at forming myotendinous junctions points to an important role of this integrin in early phases of muscle development and organization.

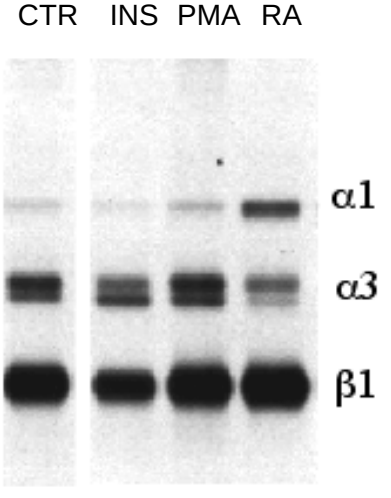

Fig. 5. Retinoic acid treatment induced increased expression of α1 integrin subunit on neuroblastoma cells. *SY5Y human neuroblastoma cells were treated as in Fig. 4 and subjected to immunoprecipitation with integrin antibodies. The position of the α and β subunits is indicated on the right side and the cellular treatment is indicated on top of each lane.*

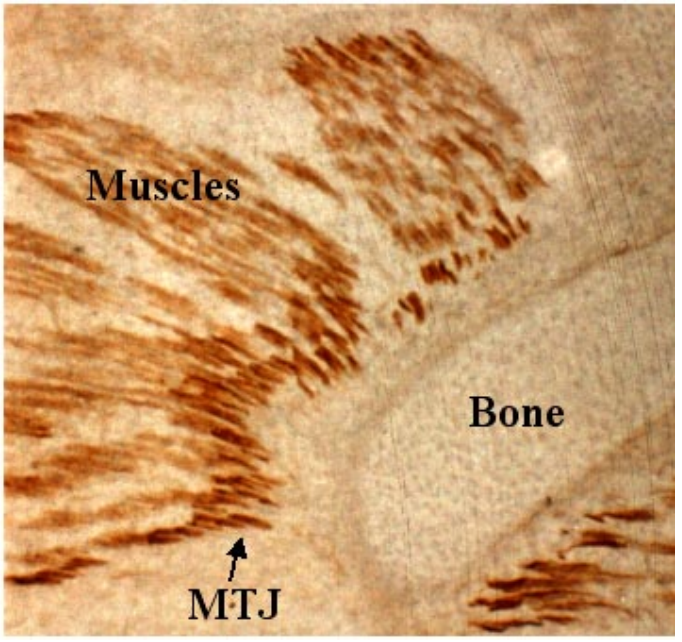

Fig. 6. Integrin αV is expressed in developing mouse muscle. *Transverse section of a mouse embryo hind leg at E15.5, stained with αV antibodies followed by peroxidase labeled secondary antibodies. Note the enrichment of αV in correspondence with the primitive myotendinous junction (MTJ).*

Another interesting change in integrin expression during muscle development concerns the β1 subunit. By performing a PCR screening of the β1 mRNA in different tissues, we and others have identified a β1 splicing isoform specifically present in striated muscle of the heart and skeletal muscles (van der Flier *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). This muscle specific isoform, named β1D, is generated by alternative splicing at the 3' end of the gene and is characterized by a distinct sequence in the last COOH terminal 24 amino acid residues of the cytoplasmic domain. We have defined the functional properties of this specific isoform by expressing it in heterologous cells that either contain the endogenous β1A (CHO cells) or lack endogenous β1 due to gene inactivation (mouse GD25 cells) (Belkin *et al.*, 1997). In both cell types β1D isoforms proved to behave as a strong adhesive molecule based on three major evidences: 1) β1D displaces β1A, the ubiquitous isoform, from sites of cell substratum adhesion when the two isoforms are coexpressed; 2) β1D interacts with the cytoskeletal protein talin more stably that β1A; 3) β1D has a higher affinity for matrix proteins compared to β1A. As results, β1D expressing cells are less motile on the extracellular matrix compared to β1A control cells and are capable to exert a stronger contractility on the adhesive substratum (Fig. 8). Based on these properties, we proposed that β1D provides a stronger link between the actin cytoskeleton and the extracellular matrix compared to the β1A isoform expressed in several different tissues. These proper-

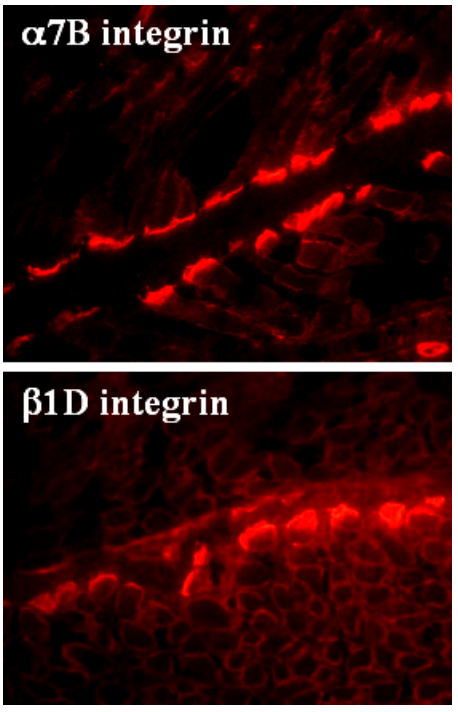

Fig. 7. The α7β1D integrin complex is enriched at the myotendinous junction in adult muscles. *Mouse hind leg muscles were cryosectioned and stained with polyclonal antibodies specific for the α7B (upper panel) or β1D integrin subunits (lower panel), followed by rhodamine labeled secondary antibodies. Note the stronger staining of the sarcolemma of myofibers in the region of tendon insertions.*

ties can explain the enrichment of the β1D at myotendinous junctions in adult muscle (see Fig. 7), where this molecule can provide a strong link necessary to withstand the mechanical tension generated during contraction.

The temporal expression of the β1D isoform during muscle development is also consistent with these functional properties (Brancaccio *et al.*, 1998). β1D, in fact, appears in skeletal muscles only in late embryos (E17.5) and its expression increases after birth to become the only integrin β1 isoform in adult muscles. An opposite pattern of expression is shown by the β1A isoform that is abundant in muscle of early embryos, but it declines and disappears from skeletal muscle a few days after birth (Brancaccio *et al.*, 1998). Thus, while, β1A represents an embryonic form of muscle integrin, β1D represents an isoform expressed from birth onward, when the mechanical loading on muscles becomes relevant. Interestingly, expression of β1D in heart follows a different pattern. In this tissue, in fact, β1D is already expressed at very early stages (E11.5) and its level remains relatively constant throughout development. This difference can be explained by the fact that heart starts pumping blood from very early stages and the mechanical strength of this muscle is important from the very beginning of embryonic life.

The different functional role of β1D and β1A is further demonstrated by *in vivo* studies by A. Sonnenberg and co-workers (Baudoin *et al.*, 1998). They modified the β1 gene by homologous recombination to generate mice strains that selectively express β1D or β1A only. Interestingly the mice that can express only β1D die early during development (E16.5) due to several defects mainly caused by altered cellular migration in the embryo. On the other hand mice expressing β1A, and lacking β1D, develop normally without gross apparent defects indicating that β1A can substitute β1D in muscle function. This finding is surprising, but does not exclude an important role of β1D in muscle function based on the following considerations: 1) β1A mice have a slightly higher level of ANP peptide in the heart indicative of a propensity to develop a hypertrophic response (Baudoin *et al.*, 1998); 2) β1D has indeed distinct functional properties from β1A as shown by expression in heterologous cells *in vitro* (Belkin *et al.*, 1997) and *in vivo* (Baudoin *et al.*, 1998) and 3) the importance of β1D might be more clearly appreciated in animals with body mass larger than mice where the mechanical loading of muscle is clearly more pronounced. As a matter of fact mice models for muscular pathologies, such as Duchenne muscular dystrophy, show a much milder phenotype compared to man in the presence of a similar genetic defect (Grady *et al.*, 1997). In this respect it will be particularly important to investigate whether mice expressing β1A would manifest muscular defects upon intense exercise and physical stress.

The cytoplasmic domain of integrins is crucial for development: in vivo analysis in transgenic mice

The results described above and several *in vitro* data indicate that the cytoplasmic domain of integrin is crucial both in linking to the actin cytoskeleton and in intracellular signaling. To investigate the importance of this function *in vivo* we have introduced mutations in the cytoplasmic domains of the α5 and β1 subunits by homologous recombination and generated mice strains homozygous for these mutations.

The α5 integrin subunit together with the β1 chain forms the fibronectin receptor. This integrin subunit is required during embryonic development as shown by the fact that mice lacking this subunit fail to develop and die around day 10.5 of embryonic life due to defect in development of mesodermal structures mostly in the posterior part of the body (Yang *et al.*, 1993). We have introduced a stop codon in the exon coding for the cytoplasmic domain to investigate the role of the cytoplasmic domain of the molecule during development. The mutation leads to a truncated α5 subunit that still retains the transmembrane segment and the membrane proximal GFFKR sequence important for correct expression at the cell surface, but lacks 18 amino acid residues at

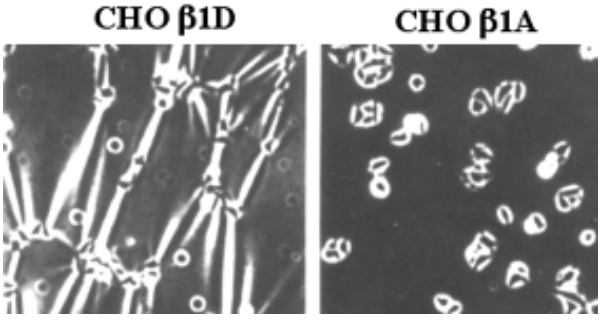

Fig. 8. Increased cellular contractility is induced by β1D integrin expression. *CHO cells expressing either human β1D or β1A integrin isoforms were plated on silicone rubber film. Note the numerous wrinkles induced on the flexible silicone substratum by β1D expressing CHO cells, indicative of a stronger contractile capacity.*

the COOH terminal (Botta *et al.*, unpublished observations). While mice heterozygous for this mutation are healthy and fertile, homozygous mice show severe defects. The penetrance of the phenotype is incomplete and approximately 50% of the mutant animals in the 129 genetic background die within the first month of life. Most of these mice have reduced weight and have a truncated and curly tail. This latter phenotype is indicative of defects in somites development, a defect similar to that observed in the α5 null mice, but restricted to the very posterior part of the body. An interesting defect observed in the homozygous mice is a reduced intestinal peristalsis as revealed by the slow speed of transit of barium bolus along the gut. The smooth muscle of the gut is one of the tissue in adult mice where α5 integrin subunit is most highly expressed. Thus it is not unlikely that α5 mutation can cause functional defect in smooth muscle contractility, which in turn could explain the reduced peristalsis. Reduced transit of the food along the gut might also cause poor intestinal adsorption and possibly explain the reduced body weight of the mutant animals.

A second mice strain was generated carrying a mutation in the cytoplasmic domain of β1 subunit. This molecule is shared by at least 12 different integrin heterodimers and its cytoplasmic domain is required for correct interaction with the actin cytoskeleton and for intracellular signaling such as activation of the cytoplasmic tyrosine kinase p125FAK (Hynes, 1992). The cytoplasmic domain of the β1 subunit consists of two structurally and functionally distinct region: the "common" and "variable" regions (Retta *et al.*, 1998). The common region consists of the membrane proximal sequence of 26 amino acids that is shared by all four splicing isoform of the molecule. The "variable region" consists of the most COOH terminal sequences defined by the four alternatively spliced forms of the mRNA and indicated as A, B, C or D isoforms. The mutation introduced by us causes a substitution of the variable region with a random sequence of 11 amino acid residues distinct from any of the four known variable sequences A, B, C or D (Hirsch *et al.*, unpublished observations). Mice carrying this mutation at homozygous level do not develop properly and die between embryonic day 10.5 and 11.5. This is an interesting finding and indicates that the truncated molecule can still support some adhesive function. In fact, mice null for β1 subunit die much earlier around day 5.5 immediately after blastocyst implantation in the uterine wall (Fassler and Meyer, 1995; Stephens *et al.*, 1995). This indicates that the β1 molecule retaining the common region of the cytoplasmic domain is capable to perform some of the adhesive function necessary for the developmental stages between day 5.5 and 11.5 of embryonic life, but fails at later stages. The major defects in homozygous mutants are pericardial swelling and intra-cardiac hemorrhage; absence of chorion-allantoic fusion and consequent lack of correct materno-fetal circulation; malformation of the neural tube which bears a kinky shape in the caudal region and may fail to close in the rostral tract.

The biochemical properties of the truncated β1 mutant have not been clarified yet and thus it is not possible at present to define the functional properties that allow the partial rescue of the β1 null phenotype nor it is completely clear which is the cause of functional failure after E11.5 stage. One possible cause of the phenotype is the defective proliferation of mutant cells. In fact, we found that cells taken from homozygous embryos and plated on culture dishes grow very poorly *in vitro* in spite of the presence of a complete cocktail of growth factors. Cells taken from wild type or heterozygous mutant littermates of the same stage of development grow normally in the same culture conditions. This suggest that one possible alteration of mutant β1 integrin is its inability to support growth. It is well established that cells need to be anchored to an extracellular matrix in order to proliferate in response to mitogens; this property is known as "anchorage dependent growth" of normal cells. The molecular basis of this process have been recently elucidated in some details and it has been shown that integrins upon interaction with the extracellular matrix trigger intracellular signals leading to activation of MAPK and JNK (Giancotti, 1997). These signals are necessary for increased synthesis of cyclin D in response to mitogens during the G1 phase of the cell cycle. One intriguing possibility is that the β1 mutant no longer activates these signaling pathways and thus is incapable to provide the anchorage signals required to respond to mitogenic stimuli. This hypothesis is currently under investigation.

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