Identification and functional analysis of genes regulated by β1 and β3 integrins

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Chapter 6:

SUMMARY AND DISCUSSION

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Integrins are heterodimeric transmembrane proteins that adhere to the extracellular matrix to mediate cell adhesion [1]. With their cytoplasmic tails integrins can bind to numerous proteins that link the integrin to the actin cytoskeleton and will initiate downstream signaling pathways as reviewed in **chapter 1**. These signaling pathways are involved in the regulation a variety of processes including migration, proliferation and differentiation [2].

While a large body of literature has been published on the effects of integrins on downstream effector proteins, less is known about the signals originating from the integrins that control gene transcription. There are several examples of genes regulated by integrins such as cdc2 whose expression is increased when β3 is reconstituted in a β3 knockout cell line [3] and uPAR whose expression is decreased after overexpression of β3 in CHO cell [4]. Moreover, when integrins interact with their ligand such as α5β1 with fibronectin or αvβ3 with vitronectin Bcl-2 expression is increased [5]. This thesis describes the identification of several genes whose transcription is regulated by integrins.

In this thesis we made extensive use of the GE11 cell line. The GE11 cell line is a neuro epithelial cell line isolated from the embryo of a β1 knockout mouse. Subsequently, human β1 has been reconstituted resulting in GEβ1 cells or human β3 has been overexpressed resulting in GEβ3 [6], [7]. Expression of β1 results in the loss of cell-cell contacts and the cells change from the epithelial-like morphology to become fibroblast-like . Additionally, these cells adhere better to fibronectin and migrate faster than the GE11 cells. On the other hand, β3 overexpression also leads to a loss of cell-cell contacts but the cells become flat and well spread with many focal contacts present over the whole basal membrane of the cell. The difference between these two cell lines has been investigated previously and it was shown that in cells expressing β1 the activity of the small GTPase RhoA is much higher than in cells in which β3 is overexpressed [7].

In **chapter 2** we analyzed the effect on gene transcription by the reconstitution of β1. Micro array analysis of the two cell lines GE11 and GEβ1 was used to identify genes whose transcription levels differed. Three genes were identified whose transcription levels were altered: that of the genes coding for thymosin- β10 and insulin-like growth factor binding protein 4 (IGFBP-4) were decreased and that of the gene coding for galectin-3 was strongly increased. Galectin-3 was chosen for further investigation because it can directly bind to integrins such as α1β1 [8] and can also influence integrin-mediated adhesion [9]. Furthermore, it is a prognostic marker for several cancers [10]. The hypervariable region of the I-like domain of β1 was shown to regulate the activity of RhoA [11] while recent observations show that it also regulates cell contractility [12]. In this study we show that galectin-3 expression is dependent on the presence of this region. However, we could not demonstrate that galectin-3 expression is dependent on the activity of RhoA. Therefore, we suggest that two separate pathways downstream of β1 regulate RhoA activity and galectin-3 expression independently. Further studies show that the increased expression of galectin-3 is important for enhanced cell adhesion to fibronectin and increased migration. While this data concurs with other reports showing that overexpression of galectin-3 increases adhesion the mode of action galectin-3 employs to enhance adhesion still remains unclear. Interestingly, in a recent paper it was shown that galectin-3 is involved in integrin-independent adhesion to the extracellular matrix through binding to the N-glycan chains attached to the matrix proteins [13]. On the other hand, galectin-3 has also been shown to mediate integrin-dependent adhesion [14], [15].

Interestingly, our data shows that galectin-3 is not involved in the maintenance of the β1-mediated cell morphology or in fibronectin fibrillogenesis, both of which are regulated by RhoA activity. Since galectin-3 expression is also not regulated by RhoA activity we conclude that galectin-3 expression and RhoA activity are regulated by separate pathways and that both in turn regulate different characteristics of GEβ1 cells.

Not only the reconstitution of β1 but also the overexpression of β3 leads to dramatic changes in cell morphology and behavior such as the loss of cell-cell contacts, increased cell spreading, migration and the formation of numerous focal adhesions [7]. Since β3 overexpression was reported to regulate uPAR expression in CHO cells [4] we investigated whether β3 overexpression in GE11 cells influences gene expression. In **chapter 3** we describe the identification of the gene coding for MRP that is downregulated when β3 is overexpressed. MRP is an appealing target of regulation by β3 since it can bind to actin

filaments [16] and can regulate the diffusion of β3 integrins on the cell surface [17], [18]. It has been shown that MRP expression can be regulated by stimuli such as LPS [19] while the expression of the close relative of MRP, MARCKS, is regulated by transformation with v-Src or p21-Ras [20]. Our investigation shows that the Ras/MAPK pathway regulates the basal expression of MRP in the GE11 cell line but β3 bypasses this pathway to downregulate MRP expression. The expression of deletion mutants of β3 showed that the last four amino acids of the cytoplasmic tail of β3 were important for MRP expression. Since this region of β3 was also shown to bind to Src [21] we tested if Src controlled MRP expression. However, this was not the case. Therefore, we conclude that a Src-independent pathway involving the binding of another protein to this region could be important for the regulation of MRP expression. Interestingly, this same region of β3 was also important for the increased cell spreading seen in GEβ3 cells. However, knockdown of MRP in GE11 or overexpression of MRP in GEβ3 cells had no effects on cell spreading, focal adhesion formation or cell morphology suggesting that MRP expression alone does not able to influence the morphology of the cells.

We continued our investigation of MRP to try to understand the mechanism MRP employs to regulate its localization within the cell. MRP uses a myristoylation motif and a polybasic cluster called the effector domain to bind to the plasma membrane in a similar way as a large group of proteins including Src, K-Ras and MARCKS. In **chapter 4** we describe how MRP localization is regulated. Controversy exists about the question how these proteins are specifically localized at the plasma membrane and not at the internal membranes that make up a larger surface area. Although it is generally accepted that these proteins are dependent on acidic phospholipids for plasma membrane targeting, it is still unknown which specific phospholipids are important. Recently, it was shown that proteins such as K-Ras use PIP_2 and PIP_3 to target to the plasma membrane [22] while a contrasting study showed that K-Ras targets to the plasma membrane because of the presence of the monovalent acidic lipid, phosphatidyl serine [23]. We show that loss of PIP_2 and PIP_3 does not affect the targeting or the association of MRP to the plasma membrane.
Therefore, we suggest that phosphatidyl serine is responsible for the targeting of MRP. Furthermore, we tested if the myristoyl-electrostatic switch model applies to the regulation of MRP localization as it does for MARCKS. This model predicts that the combination of the binding of the myristoyl moiety and the positively charged effector domain are needed for the association of the protein with the membrane [24]. From our data we can conclude that this model applies for the association of MRP with the plasma membrane.

MRP associates with the plasma membrane but upon phosphorylation of the serine residues within the effector domain the protein is translocated away from the plasma membrane to internal membranes such as those of the lysosomes in a matter of seconds. Unlike MARCKS whose translocation from the plasma membrane to the internal membranes only occurred after several hours [25] MRP was so rapidly translocated to internal membranes that it was not possible to detect an increase in cytoplasmic MRP using fractionation assays. When the effector domain was deleted from the protein, MRP was no longer able to bind to the plasma membrane but was still present on the internal membranes. This raises the question how MRP associates with these internal membranes under these circumstances. If the myristoyl-electrostatic switch model also applies to the binding of MRP to internal membranes it would be impossible for MRP to bind when the effector domain is deleted. Therefore, the model explains the association of MRP to the plasma membrane through the cumulative effect of the myristoyl moiety and the effector domain binding to the plasma membrane but we suggest that MRP only utilizes the myristoyl moiety for association with internal membranes. Thus, the effector domain functions primarily as a plasma membrane targeting motif.

In different cancers an increase in c-Src activity has been observed even though activating mutations in the *SRC* gene are rare. While this argues against a role for c-Src in tumor initiation, it has been implicated in the formation of metastases and tumor growth. In **chapter 5,** it is shown, that the expression of "primed" c-Src carrying a mutation at the tyrosine 530 that disrupts intramolecular association between this residue and the Src homology domain 2 (SH2), in cells expressing β3, but not β1, promotes tumorigenicity. The cooperation between αvβ3 and c-Src seems to be important since increased αvβ3 expression has been linked to growth and tumor progression in cancers in which c-Src activity is often increased. In this chapter we show that αvβ3 and primed c-Src are functionally linked to promote tumorigenicity in the cell lines tested. Others showed that there is a direct association between the SH3 domain of Src and the C-terminal YRGT motif of β3 [21]. We found that the presence of this YRGT motif of β3 is essential for the increased tumorigenicity.

Moreover, Src activity is also increased in cells expressing β3. Src is activated in a stepwise process. Clustering of αvβ3 brings the associated primed Src molecules in close contact with one another allowing their autophosphorylation in trans resulting in their full activation. Therefore, we conclude that the binding of Src to β3 allows the clustering of Src resulting in the activation of Src leading to increased cell growth and tumorigenicity.

To conclude, we have identified several new targets of regulation by integrins and investigated their role in the integrin-mediated effects observed in our cell system. Furthermore, we have shed light on the mechanism by which MRP is targeted to the plasma membrane and how its association with this membrane is regulated. Lastly, we have shown that there is a functional link between Src and β3 during tumorigenesis.

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