Tetraspanin CD9 in cell migration

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Abstract
Tetraspanin CD9 is associated with integrin adhesion receptors and it was reported that CD9 regulates integrin-dependent cell migration and invasion. Pro- and anti-migratory effects of CD9 have been linked to adhesion-dependent signalling pathways, including phosphorylation of FAK (focal adhesion kinase) and activation of phosphoinositide 3-kinase, p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase). In the present paper, we describe a novel mechanism whereby CD9 specifically controls localization of talin1, one of the critical regulators of integrin activation, to focal adhesions: CD9-deficiency leads to impaired localization of talin1 to focal adhesions and correlates with increased motility of breast cancer cells.

Tetraspanins and integrins
Cell migration on ECM (extracellular matrix) ligands is a complex process that is co-ordinated at various levels and relies on a multitude of spatially separated protein–protein interactions. Integrins are the principal heterodimeric receptors for ECM ligands, and integrin complexes play a pivotal role in the migratory process [1,2]. The ligand-binding and signalling functions of integrins are controlled through a network of associated transmembrane and cytoplasmic proteins [3]. We and others have previously established that tetraspanins, a family of four-transmembrane domain proteins, are associated with integrin receptors and regulate various aspects of integrin functions [4,5]. These include integrin activation, ligand binding [6] and adhesion-dependent signalling [4], and it has also been reported that tetraspanins regulate the glycosylation [7] and endocytosis [8] of integrins.

The role of CD9 in cell motility
Tetraspanin CD9 was first described as a motility-related factor in 1991 when it was reported that specific anti-CD9 mAbs (monoclonal antibodies) inhibited the migration of multiple cancerous cell lines [9,10]. Subsequently, it was established that CD9 is associated with various integrins, including α3β1, α4β1, α5β1, α6β1, α6β4 and αIIbβ3 [5], and pro- and anti-migratory functions of CD9 were attributed to its modulatory activity towards integrin complexes. The proposed mechanisms involved CD9-dependent changes in conformation and activation of integrins [11], modulation of integrin-dependent signalling pathways including phosphorylation of FAK (focal-adhesion kinase) [12], activation of phosphoinositide 3-kinase and phosphorylation of p130Cas [11], an adaptor protein that plays a critical role in integrin signalling [13]. More recent results suggested that, in addition to modulating integrin function, CD9 has the potential to regulate motility through other transmembrane proteins. Indeed, it was reported that CD9 is directly associated with proteins of the EWI family [14], EGFR [EGF (epidermal growth factor) receptor] [15] and DDR1 (discoidin domain receptor 1) [16]. Importantly, each of these newly identified CD9 partners can play an important role in regulating cell motility on various ECM ligands. For example, interactions of CD9 with EWI proteins appear to regulate clustering of integrins at the plasma membrane [17], a critical process during cell migration. In addition, EWI proteins (and, therefore, CD9) may contribute to integrin-dependent reorganization of the actin cytoskeleton via ezrin, an actin-binding ERM (ezrin/radixin/moesin) protein [18]. CD9 appears to destabilize surface expression of EGFR, and consequently, attenuates ligand-induced activation of the receptor [15]. Thus it is likely that CD9 also affects EGFR-dependent chemotactic migration. DDR1 is a tyrosine kinase receptor for collagens, and it has been shown that knockdown of either DDR1 or CD9 inhibits migration of breast cancer cells on collagen [16]. Finally, it has been reported that the large extracellular loop of CD9 directly interacts with fibronectin, and the authors proposed that the CD9–fibronectin interaction is required for increased haptotactic migration of CHO (Chinese-hamster ovary) cells overexpressing this tetraspanin [19].

Depletion of CD9 increases proliferation of breast cancer cells in 3D (three-dimensional) ECM
The MDA-MB-231 breast cancer cell line is widely used for investigating molecular mechanisms of tumour cell migration, invasion and metastasis. Importantly, in our earlier studies,
we showed that anti-CD9 mAb induced invasive protrusions when MDA-MB-231 cells were cultured in 3D ECM [20], thereby implicating CD9 in the migration of breast cancer cells. Because this approach did not allow unambiguous interpretation of the observed phenotype (indeed, it is not known whether anti-CD9 mAbs used in the early experiments inhibited or activated protein function), we decided to examine the role of CD9 in cell motility more directly by investigating how depletion of this tetraspanin might change the migratory behaviour of MDA-MB-231 cells. To this end, we generated MDA-sh4 and MDA-sh5 cell lines in which the expression of endogenous CD9 was stably decreased by >95 and ∼80% respectively (Figure 1A). Both cell lines were established after sorting for CD9− cells from puromycin-resistant bulk populations. Detailed flow cytometric analysis showed that the depletion of CD9 did not change the surface expression of other tetraspanin proteins (CD63, CD81, CD82 and CD151) or various integrin subunits (α3, α5, α6 and β1). Moreover, under standard culture conditions (i.e. cells grown on tissue culture plastic), we found that down-regulation of CD9 neither had any apparent effect on cell morphology nor did it change the proliferative rate of MDA-MB-231 cells. We then examined the growth of the cells in 3D Matrigel™ and polymerized collagen type I. Interestingly, proliferation of MDA-sh4 and MDA-sh5 cells was enhanced within both matrices when compared with MDA/cont, CD9+ MDA-MB-231 cells (Figure 1B). In contrast, the depletion of CD9 had no apparent effect on the morphology of ECM-embedded single cells (or resultant cellular aggregates). Both CD9+ and CD9− MDA-MB-231 cells formed compact round colonies in 3D Matrigel™: the number of colonies and their overall shape were comparable for all cell lines. In 3D collagen, MDA-MB-231 cells exhibit an elongated morphology and, in time, the parallel alignment of proliferating cells resulted in the formation of a cellular network; depletion of CD9 had no apparent effect on the kinetics of network development and their overall pattern. Thus we conclude that, while the presence of CD9 has a suppressive effect on the growth of breast cancer cells in 3D ECM, the invasive behaviour of the cells was not affected after CD9 depletion.

Depletion of CD9 inhibits cell spreading and facilitate cell migration
To investigate further how CD9 might influence integrin function in breast cancer cells, we carried out adhesion, spreading and migration experiments. A standard 30-min static adhesion assay showed that depletion of CD9 did not affect the attachment of serum-starved cells to fibronectin, collagen I or Matrigel™. Knockdown of CD81, a closely related tetraspanin, also had no effect on adhesion of MDA-MB-231 cells to these substrates. Short-term spreading experiments (30 and 45 min after plating) revealed that the area occupied by CD9− cells was ∼1.5-fold larger when compared with control CD9+ cells. The differences in spreading were
negated when cells were allowed to spread on fibronectin for more than 6 h. A standard Boyden chamber migration assay was performed to examine the involvement of CD9 in motility of breast cancer cells. These experiments showed that the migration of MDA-sh4 and MDA-sh5 cells towards fibronectin (in either the presence or absence of chemotactic stimulus) was increased by 1.5–2.5-fold when compared with CD9+ cells (Figure 1C). Importantly, when the expression of CD9 was reconstituted in MDA-sh4 and MDA-sh5 lines (MDA-sh4/CD9rec. and MDA-sh5/CD9rec. cells respectively), the phenotype of cells reverted to being less migratory. Because the expression levels of $\alpha_5\beta_1$ integrin, a principal fibronectin receptor in MDA-MB-231 cells, were comparable in all cell lines, our results show that the presence of CD9 has a negative effect on the pro-migratory function of this integrin.

**CD9 regulates recruitment of talin1 to focal adhesions**

To investigate the molecular mechanisms underlying the inhibitory effect of CD9 on $\alpha_5\beta_1$-dependent cell spreading and migration, we examined the assembly of focal adhesions in cells plated on fibronectin. Initially, we found that the distribution of vinculin, paxillin and FAK were comparable in CD9+ and CD9− cells (Figure 2A, upper panel). We also observed no noticeable differences in the number of focal adhesions positive for these proteins. In contrast, the depletion of CD9 resulted in decreased localization of talin to focal adhesions (Figure 2A, lower panel). Concurrently, we detected an increase in talin cytoplasmic staining in CD9− cells. In these experiments, we used the mAb 8d4. Although this antibody detects talin1 and talin2 (two isoforms of talin which are known to have both unique and redundant functions) equally well by Western blotting, we have consistently observed that its reactivity towards talin1 was notably better in immunofluorescence experiments. Hence, these results suggested that CD9 may primarily affect the distribution of talin1. Indeed, using isoform-specific mAbs we established that the depletion of CD9 specifically changed the distribution of talin1 (Figure 2B). On the other hand, the total levels of intact and proteolytically cleaved talins were comparable in CD9+ and CD9− MDA-MB-231 cells. To quantify CD9-dependent differences in the recruitment of talin to focal adhesions we co-stained cells with anti-FAK and anti-talin (8d4) antibodies; ImageJ was then used to quantify the size and number of focal adhesions for each of the antibodies by thresholding the 8-bit images and measuring particles between 0.3 and 70 $\mu$m$^2$. This was achieved by altering brightness and contrast levels to highlight staining corresponding to focal adhesion-like structures; focal adhesions were classified as anything over a particular size (i.e. five adjoining pixels). As illustrated in Table 1, there was a $\sim$2.5-fold decrease in the number of focal adhesions which were positive for both FAK and talin in MDA-sh4 cells: i.e. the ratio of focal adhesions positive only for FAK was $\sim$2.5 higher. Furthermore, we observed an increase in the ratio of the total area occupied by FAK-positive focal adhesions to that occupied by talin-positive focal adhesions in CD9-deficient cells. Importantly, these trends were reversed when MDA-sh4 cells were reconstituted to express CD9 (in MDA-sh4/CD9rec. cells). We have previously reported that talin is co-localized with various tetraspanin proteins (including CD9) in the primordial adhesion complexes [22]. Thus it is possible that depletion of CD9 may lead to destabilization of integrin–talin interactions.

**Conclusions**

We have shown that the depletion of CD9 increases $\alpha_5\beta_1$-mediated migration of breast cancer cells towards fibronectin. These results along with previously published results [12,23–25] firmly establish the anti-migratory function of CD9. Although previous reports suggested that the inhibitory role...
of CD9 in cell migration might be linked to the suppression of FAK [12] or PI3K (phosphoinositide 3-kinase) [26], our results point to an alternative mechanism. Indeed, we found that adhesion-dependent activation of FAK and c-Akt, a downstream target for PI3K, was not affected in CD9- MDA-MB-231 cells. Instead, our results indicate that CD9 may be involved in the recruitment of talin1 to focal adhesions. Interestingly, CD9 does not seem to control focal adhesion localization of several other cytoskeletal signalling proteins in MDA-MB-231 cells, including talin2. Given that talin2 can functionally substitute for talin1 with regard to focal adhesion assembly and cell spreading in cultured cells [21,27], it seems likely that talin2 is responsible for the localization of vinculin, FAK and paxillin to focal adhesions in CD9- cells.

What are the possible mechanisms of CD9-dependent differential recruitment of talins to focal adhesions? We have previously showed that, in contrast with many other focal adhesion components, talins are localized in CD9-containing primordial adhesion complexes [22]. Thus CD9 may function as an accessory protein controlling a molecular switch between integrin–talin1 and integrin–talin2 complexes at the initial stages of cell spreading and establishing integrin-based adhesion structures. Alternatively, CD9 may control the cellular distribution of talin1 indirectly. For example, CD9 forms complexes with phosphatidylinositol 4-kinase class II [28], an enzyme involved in the biosynthesis of PIP2 (phosphatidylinositol 4,5-bisphosphate), a known activator of talin1 [29]. Interestingly, there is a difference in three amino acids between talin1 and talin2 in one of the PIP2-binding regions (residues 370–378). Although the functional significance of this variation is currently unknown, this gives a potential scope for differential regulation of talins by CD9 through phosphoinositides.

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References


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