Chinese Hamster Ovary Cell Motility to Fibronectin Is Modulated by the Second Extracellular Loop of CD9

IDENTIFICATION OF A PUTATIVE FIBRONECTIN BINDING SITE*


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CD9, a member of the tetraspanin family of proteins, is characterized by four transmembrane domains and two extracellular loops. Surface expression of CD9 on Chinese hamster ovary (CHO) cells dramatically enhances spreading and motility on fibronectin. To elucidate the mechanistic basis of CD9-fibronectin interaction, binding to fibronectin was investigated using purified and recombiant forms of CD9. The affinity of fibronectin for CD9 in enzyme-linked immunosorbent assay was 81 ± 25 nM. The binding of fibronectin to immobilized CD9 was enhanced by Ca²⁺ ions. Protein binding and peptide competition studies demonstrated that peptide 6 derived from CD9 extracellular loop 2 (amino acids 168–192) contained part of the fibronectin-binding domain. Additionally, enhanced adhesion of CD9-CHO-B2 cells to fibronectin was significantly reduced by peptide 6. CD9-CHO cells had a 5-fold increase in motility to fibronectin as compared with mock-transfected controls, an effect that correlated with CD9 cell surface density. Truncation of CD9 extracellular loop 2 and peptide 6 caused inhibition of CD9-CHO cell motility to fibronectin. Deletion of CD9 extracellular loop 1 had no significant effect on CHO cell motility. These findings demonstrate a critical role for CD9 extracellular loop 2 in cell motility to fibronectin and clarify the mechanism by which CD9-fibronectin interaction modulates cell adhesion and motility.

The transmembrane 4 superfamily (TM4SF) member, CD9, is a 24-kDa integral membrane glycoprotein expressed on numerous cell types, including platelets, endothelial cells, smooth muscle cells, cultured fibroblasts, pre-B cells, activated T cells, and glial cells (1). Based on cDNA sequence analysis, the TM4SF members are predicted to be single polypeptide chains with four highly hydrophobic putative transmembrane (TM) regions and two extracellular (EC) loops with both the NH₂ and COOH termini localized intracellularly. The putative transmembrane domains and certain residues in the EC loops are highly conserved, suggesting that these proteins perform closely related functions (1). The cellular function of the TM4SF proteins is not yet clear, but indirect data suggest that most TM4SF members mediate cellular functions such as adhesion, motility, and differentiation (1).

The role of CD9 in many cell types has been investigated via anti-CD9 mAb perturbation studies. Anti-CD9 mAbs have been shown to mediate the proliferation, adhesion, and motility of neural cells (2–4). An anti-CD9 mAb enhanced the migration of Schwann cells on living neurites and sciatic nerve sections (2). Antibody-mediated enhancement of Schwann cell migration correlated with increases in cytosolic calcium and phosphoproteins. Ectopic expression of CD9 on nonmotile Raji cells gave rise to β₁ integrin-dependent motility on FN (5). CD9 was also found to participate in endothelial cell migration during wound repair (6). Recent studies have shown that CD9 deficiency in mice does not significantly affect smooth muscle cell migration or neointima formation after vascular injury (7). However, in the absence of CD9, one or more of the other TM4SF members may compensate for lack of CD9 function. Conversely, studies using CD9-null mice have demonstrated an essential role for CD9 in egg/sperm fusion (8, 9). Previous studies have shown that high affinity binding between fertilin beta (ADAM2) and α₅β₃ integrin requires cooperation between α₅β₃ and CD9 (10).

Immunoprecipitation studies on a number of cell types have shown that CD9 and other TM4SF members associate with integrins (1). CD9 was specifically coimmunoprecipitated from S-16 Schwann cell extracts using mAbs against integrins α₅, α₆, and β₁ and double-immunofluorescence labeling studies suggested that CD9 colocalizes with these integrins on the cell membrane (11). Recent studies have shown that prototype complexes of CD9, CD81, and α₅β₁ and complexes of CD63 and phosphoinositide 3'-OH kinase may localize within lipid raft-like domains on the cell surface (12). These data suggest that CD9 either interacts directly with ECM proteins or influences the activity of adhesion molecules indirectly via physical association or the modulation of intracellular signaling pathways.

We have previously demonstrated that surface expression of CD9 in CHO cells induced unique growth patterns and a dramatic enhancement of CHO cell spreading on the extracellular matrix protein, FN (13). In this study, we showed that binding...
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of purified FN to platelet-derived or recombinant CD9 was dose-dependent, enhanced by Ca\(^{2+}\) ions, and that the affinity of this interaction was 80 nm. Part of the FN-binding domain was located within CD9 EC2 (residues 168–192). A peptide spanning this EC2 region competitively inhibited the interaction of the CD9 with FN in a purified ELISA system and inhibited the adhesion of CD9-CHO-B2 cells to FN. We also showed that CHO cells with high CD9 surface density had significantly greater FN-directed cell motility as compared with mock-CHO cells. These effects were seen with two independent CHO cell clones and with two heterogenous populations of CD9-expressing CHO cells. Using CD9-CHO cells expressing a lower surface density of human CD9, we were able to demonstrate that CD9 surface density had a direct relationship with CHO cell motility to FN. Mutant CD9-CHO cells lacking regions of CD9 EC2 had significantly decreased haptotactic motility to FN. Additionally, CD9-mediated CHO cell motility was specifically blocked by peptide 6 corresponding to the FN binding site of CD9. Deletion of CD9 EC1 did not significantly affect CD9-mediated motility. These findings suggest that CD9-FN interaction markedly influences FN-directed CHO cell movement and that direct interaction between CD9 and FN is required for this effect.

EXPERIMENTAL PROCEDURES

Materials — The wild type CHO cell line (CHO-K1 ATCC #CCL-61) was purchased from the American Type Culture Collection, Rockville, MD. The CHO-B2 cell line, deficient in αβ chain expression, was kindly provided by Dr. R. Juliano of the University of North Carolina, Chapel Hill, NC (14). Peptide 5 (YKDTYNYLKTKEPQRTLKLAKA), peptide 6 (PDKDVLETSFVSCPDIAKVFVDKN), and peptide 6S (KEFDFKA), provided by Dr. R. Juliano of the University of North Carolina, Chapel Hill, NC, were either prepared and purified by Dr. Jerome Seyer and Dr. Bob Cassell, Veteran PSVCKVEDIDTKTLPKVN) was expressed and purified from pCMVCD9 (bp 153–893) had been described previously (17). A CHO cell clone transfected with pCMVCD9 had been described elsewhere (13). The oligonucleotide primers used to construct the CD9 EC2 truncation cDNAs were designed according to the reported CD9 nucleotide sequence (17). These oligonucleotide primers were used in PCR amplifications using full-length CD9 cDNA as a template to generate mutant CD9 cDNAs. Three PCR amplifications were used to construct CD9 EC2 deletions. The first PCR was done using full-length CD9 cDNA as a template with a CD9 SpH1 5’ primer (5’-CAGTCGACTGTGCCGTTGTTTGTTCCGCTCTT-3’) containing the SpH1 site at position +416 in the CD9 open reading frame with either 3’ Δ133–192 (5’-TCGGCGGGATTGTGGGAAACAGCTGTTTGTTAGTT-3’), 3’ Δ152–192 (5’-GGCGGTATGAGGCTAGTTGGAATACTCTTTCTCTTGGG-3’) primers generating 154-, 208-, and 271-bp fragments, respectively.

The second PCR was done using full-length CD9 cDNA as a template with a 3’ ApaI primer (homologous to the pReCMV vector backbone sequence) with either 5’ Δ133–192 (5’-ACTACAAAGACGTCGCTGCT-3’), 5’ Δ152–192 (5’-CACTATCGCTGTTAGTCTTCACCATCGG-3’), 5’ Δ175–192 (5’-CCGAAGAGAGCTACAGGATCT-3’) primers, respectively, generating a 611-bp fragment in each case. For the third PCR amplification, corresponding overlapping PCR products were utilized as templates and extended for 15 cycles, after which CD9 SpH1 and ApaI primers were used for an additional 30 cycles to generate CD9 EC2 internal deletion products of 785 bp for Δ133–192, 819 bp for Δ152–192, and 882 bp for Δ173–192. These PCR products were cleaved with SpH1 and ApaI and subcloned into the pBSKCD9 vector backbone from which the SpH1/ApaI portion of the CD9 cDNA/vector sequence had been removed generating complete CD9 cDNAs with the targeted regions in CD9 EC2 missing. The CD9 EC2 truncation cDNAs had been subcloned into the original pRe/CMV vector construct from which the full-length CD9 cDNA had been removed. The molf DNA sequencing system (Promega, Madison, WI) was used to obtain and confirm CD9 EC2 truncation cDNA sequences. In summary, Δ133–192, Δ152–192, and Δ173–192 CD9 cDNAs had truncations of 180 bp (60 aa), 123 bp (41 aa), and 60 bp (20 aa) in CD9 EC2, respectively.

To construct Δ323 CD9 cDNA with an internal deletion of 72 nucleotides (24 aa) spanning CD9 EC1, a 688-bp BamHI/PstI fragment of CD9 encoding cDNA was amplified using pRe/CMVCD9 as a template using DAWfor (5’-GGACTCTGTCGCTGCTCAGAGG-3’), and DAWrev (5’-CCTAGCATGAGCACCCATGCGCTCAG-3’) primers. This fragment was digested with BamHI and PstI and cloned into complementary sites in the vector pGEM-T (Promega, Madison, WI) yielding the pGDAW construct. To delete CD9 EC1, pGDAW was amplified using DAWfor and
Δ23rev (5'-GACTCTGTCCATAGTCCAAAT-3') primers. The Δ23rev primer contained a T187,C254 nucleotide junction in the CD9 sequence. 5 μl of the first PCR product was mixed with 1 ng of pGDAW plasmid and subjected to one PCR cycle of 5 min at 95 °C, 2 min at 37 °C, and 10 min at 70 °C. The product of the second PCR was then amplified for 30 cycles using Δ23rev and ΔArev primers generating the 617-bp BamHI/PstI CD9 EC1 deletion fragment. The CD9 EC1 deletion product was digested with BamHI and PstI and cloned into the complementary sites of the plasmid pGEM-T thus generating pGAW/D35. The pGAW/D35 plasmid was grown and purified from E. coli. A 617-bp BamHI/PstI fragment from pGAW/D35 was isolated and subcloned into the BamHI/PstI site of pCDNA3.1-Zeo to generate pCDNA/Δ23.

The REP4CD9 expression vector was made by amplification of CD9 cDNA by PCR using pRC/CMVD9 as a template and primers CD9for (5'-GATCAGCTTTAGCAGTAAAGG-3') and CD9rev (5'-GATCTGGATCCCTAGGATCCCCG-3') containing HindIII and BamHI restriction sites, respectively. The resulting DNA amplified by PCR was digested with HindIII and BamHI and cloned homologous sites of the pREP4 vector.

Cell Transfections—Wild type CHO cells were grown to 50–70% confluency in six-well tissue culture plates (Corning Glass, Corning, NY) containing 3 x 10^5 cells/well. Cells were rinsed once with serum-free RPMI 1640 and transfected with 2 μg of plasmid DNA using LipofectAMINE according to the manufacturer's protocol. At 72 h post-transfection, cells were passaged 1:10 in selective growth media supplemented with 750 μg/ml Geneticin G418 or 1 mg/ml Zeocin, and stable transfectants were selected. Two mock control transfections were done, “Mock” for pRC/CMV and “Mock-Zeo” for pCDNA3.1-Zeo. The heterogeneous populations of CD9-expressing CHO cells CD9-CHO-H1, CD9-CHO-H2, and REP4-C9-CHO were made by electroporation with either pRC/CMVD9 or pREP4CD9 for REP4/C9-CHO cells. Briefly, washed CHO cells were resuspended at 5 x 10^7 in PBS, mixed with 20 μg of the appropriate plasmid DNA, and pulsed at 500 V, 900 microfarads, and 120s. DNA/RNA electroporation into CHO cells was performed as described (data not shown).

Cell Culture—Cell cycle-synchronized cultures were used for all assays. CD9- or Mock-CHO cells were enriched at the G0/G1 stage by cell synchronization, harvested, and prepared as described for Boyden chamber motility experiments. 4 x 10^5 CHO cells/ml was added per coated tissue culture insert. CHO cells were rinsed once with serum-free RPMI 1640, transfected with 2 μg of plasmid DNA using LipofectAMINE according to the manufacturer’s protocol. At 72 h post-transfection, cells were passaged 1:10 in selective growth media supplemented with 750 μg/ml Geneticin G418 or 1 mg/ml Zeocin (Invitrogen) depending on expression vector selection marker. The heterogenous CHO cell line transfected with pREP4CD9 was grown in media A supplemented with 400 μg/ml hygromycin for pREP4CD9-transfected CHO cells.

Motility Assays—The motility assays were performed using two methodologies. The first method was modified from Bauer et al. (18). Cell cycle-synchronized CHO cells were seeded into tissue culture plates, grown to ~50% confluency, and harvested. The cells were washed twice with media A, adjusted to 4 x 10^5/ml and grown to 100% confluency in media A. The second method for assessing the motility of CD9-transfected CHO cells utilized 10-mm polycarbonate tissue culture inserts with 8-μm pores (Nunc, Rochester, NY). Tissue culture inserts were coated on the underside with FN as described above for Boyden chambers. For peptide inhibition studies FN-coated tissue culture inserts were coated with either peptide or scrambled peptide control (referred to as 6S) at 2 μg/ml (8.1 μg/ml) and blocked with PBS, 3% BSA. CHO cells were cell synchronized, harvested, and resuspended at 1 x 10^5 cell/ml in adhesion media (RPMI, 1% BSA). For peptide inhibition studies, peptides were added to CHO cell suspension to give a final concentration of 0.5 μM, and cells were incubated at 37 °C for 30 min. 1-ml CHO cell suspension was added to each well, and CHO cells were allowed to adhere for either 3 or 6 h when adherent cells were stained with Wright Giemsa and counted.

RESULTS

CD9 Binding to Fibronectin Is Calcium-dependent—As previous studies had suggested a role for CD9 in cell adhesion and spreading on FN (13), we assayed purified CD9 for FN binding activity. The interaction between CD9 and FN was stimulated by CaCl2 (Fig. 1A). Although other cations, including Na+, and K+, had no effect on CD9-FN interaction. Mg2+ showed some enhancement of CD9-FN binding (data not shown). Half-maximal binding was seen at ~1 μM CaCl2. The counterion did not appear to influence the effect of Ca2+ on CD9-FN binding (data not shown). In the presence of 2.5 μM Ca2+, half-maximal binding of soluble FN to immobilized CD9 was seen at 35.6 ± 11 μg/ml (8.1 ± 2.5 x 10^-8 M) FN. Representative CD9-FN

FIG. 1. FN can directly bind to CD9. A, ELISAs were used to evaluate the effects of increasing concentrations of CaCl2 on the binding of FN (100 μg/ml) to immobilized CD9. Closed and open symbols represent data from two different experiments. B, the relative binding of FN (0–100 μg/ml) to purified platelet CD9 (closed circles), His6-rCD9 (open circles), and BSA (open triangles) were compared by ELISA. 2.5 μM Ca2+ was included in the assays.

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interactions in the presence of 2.5 mM CaCl$_2$ are shown in Fig. 1B. In the absence of Ca$^{2+}$ ions, the binding is lower, and saturation is not reached at 200 µg/ml FN (data not shown).

The binding of FN to purified platelet CD9 was compared with bacterially expressed histidine tagged CD9 (His$_6$-rCD9) and BSA (Fig. 1B). The absolute values from ELISA showed that the binding of His$_6$-rCD9 to FN was 40% lower than for purified CD9; however, half-maximal binding between His$_6$-rCD9 and FN was 120 nM FN, close to the value obtained for purified CD9 (81 ± 25 nM).

To address the specificity of the CD9-FN interaction, peptides corresponding to different regions of CD9 EC2 were assayed for FN binding activity (Fig. 2A). Peptide 5 (YKDTYNKLKDEPQRETLKAI) and Peptide 6 (PKKVDELETFVKSCPDAIKEVFDNK) correspond to CD9 EC2 amino acids 125–146 and 168–192, respectively. When compared with intact platelet-purified CD9, soluble FN had significant binding to peptide 6 (65%) compared with 19% for peptide 5. Competition ELISAs were used to examine the effects of the CD9 peptides on the binding of FN to CD9 (Fig. 2B). Peptide 6 significantly inhibited the binding of FN to CD9 where maximal inhibition was seen at 60 µM peptide (Fig. 2C). To demonstrate that CD9 was able to bind to FN in a whole cell system, CHO-B2 cells lacking integrin $\alpha_5\beta_1$ expression were transfected with CD9. The ability of CD9-CHO-B2 cells to adhere to FN was compared with Mock-CHO-B2 cells. CD9-CHO-B2 cells had increased adhesion to FN in the absence of integrin $\alpha_5\beta_1$ expression when compared with Mock-CHO-B2 cells. Peptide 6 inhibited the adhesion level of CD9-CHO-B2 cells on FN to that seen with Mock-CHO-B2 cells demonstrating that CD9 was directly responsible for the enhanced adhesion of CD9-CHO-B2 cell to FN. A scrambled control (peptide 6S) had no significant effect on CD9-CHO-B2 cell adhesion ($p > 0.005$).
CD9 Expression Enhances CHO Cell Motility to Fibronec-
tin—The CD9-CHO-N3 clonal cell line was isolated from CHO
cells transfected with pRc/CMVCD9 (17). Polycarbonate filters
coated with either fibrinogen or BSA had no adhered CD9-
CHO-N3 cells in motility assays after 6 h (data not shown). The
CD9-CHO-A6 clone described in this study was derived from
CHO cells transfected with pRc/CMVCD9. Both CD9-CHO-N3
and CD9-CHO-A6 cell clones had high surface expression of
CD9 as demonstrated by flow cytometry. For example, over
93% of CD9-CHO-A6 cells expressed CD9 with a mean fluores-
cence intensity (MFI) of 760 on labeling with mAb7 compared
with Mock-CHO cells with an MFI of 6.7. To demonstrate that CD9
effects on CHO cell motility were not due to aberrant
clones, two clonally heterogenous populations of CD9-express-
ing CHO cells were generated. Both CD9-CHO-H1 and CD9-
CHO-H2 had the same enhancement of CHO cell motility to FN as
seen with CD9-CHO-A6 (Fig. 3), demonstrating that aberrant CHO cell clones
were not responsible for the changes in CHO cell motility.
Additionally, CD9-CHO-REP4 had a 45% reduction in hapto-
tactic motility to FN as compared with the other CD9-express-
ing CHO cell lines (Fig. 3). These data point to a direct rela-
tionship between CD9 cell surface density and the
enhancement of CHO cell motility to FN. Overall, we conclude
that CD9 expression on CHO cells is associated with increased
haptotactic motility to FN.

CHO Cells Expressing CD9 Truncations Had Decreased Hap-
totactic Motility to FN—In this study, we have shown that FN
can directly bind to CD9. Peptide 6 corresponding to position

FIG. 4. Effects of CD9 extracellular loop deletions on the CD9-mediated haptotactic motility to FN. A, motility of CHO-K1 cells transfected with either Mock-, CD9-, or CD9 EC2 deletion mutants to FN was measured using the Boy-
den chambers as described under “Exper-
imental Procedures.” Removal of CD9 EC2, TM4, and COOH-terminal resulted in a 58% reduction in motility. Cells ex-
pressing CD9 internal deletions of aa residues 133–192 and 152–192 demonstrat-
ed a 60 and 64% reduction in motility, respectively, over 6 h (p < 0.05). In addition, cells transfected with a CD9 truncation containing a part of the FN binding site (CD9 Δ173–192) exhibited a reduc-
tion of motility of 45%. B, the motil-
ity of CHO-K1 cells expressing CD9 EC1 deletion mutant (Δ23) was compared with the Mock transfection (Mock-Zeo) and
CHO-CD9-A6 expressing full-length CD9. Deletion of CD9 EC1 has no significant effect on CD9-mediated CHO cell motility on FN at 6 h (p > 0.005). C, peptide 6 corre-
sponding to the putative FN binding site on CD9 EC2 (aa 168–192) competitively inhibited CD9-mediated CHO cell motility to FN (p < 0.005). The specificity of this effect was shown by a lack of inhi-
bition with a scrambled Peptide 6 (Pepti-
dle 6S).
168–192 in CD9 EC2 competitively inhibited binding of FN to purified CD9 in a dose-dependent fashion (Fig. 2C). Therefore, we hypothesized that this region on CD9 EC2 could be important in mediating FN-directed motility of CD9-CHO cells.

To test whether the FN binding site is required for CD9-mediated cell motility, we constructed several CD9 deletion mutants. Four CD9 truncations were located in CD9 EC2. CD9Δ113–228 contained a 114-aa deletion encompassing the whole EC2 loop, the fourth TM domain, and the third cytoplasmic domain. Δ133–192 contained an internal deletion of 60 aa, and CD9Δ152–192 comprised a 41-aa deletion. These EC2 deletions included the entire putative FN-binding region (aa 168–192). CD9Δ173–192 contained a 20-aa deletion of CD9 EC2 spanning part of the FN-binding region. Five amino acids (PKKDV) at the NH2-terminal portion of the FN-binding region were not removed in this deletion. CD9Δ23 truncation encompassed the entire first loop of CD9. CD9 cDNAs were subcloned into expression vectors pRc/CMV or pCDNA3.1Zeo and stably transfected into CHO cells. Transfection of WT CD9 cDNA was performed as an internal control. For each transfection, twen-
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ty-four clones were selected and expanded for further characterization. The CHO cell clones expressing mutant CD9 had equivalent surface density compared with WT CD9 when used as an internal control (19). Northern blot analysis also confirmed a comparable expression of mutant CD9 RNA when compared with the CD9-expressing CD9 N3 clone (19). Earlier studies have shown that the CD9 EC2 truncation Δ113–228, lacking CD9 EC2, TM4, and the COOH-terminal, can be detected on the surface of CHO cells at equivalent density to full-length CD9 using the CD9 EC1-specific polyclonal antibody RAP2 (13). CHO cell clones containing CD9 truncations were analyzed for haptotactic motility to FN. CHO cells expressing CD9 truncations within the FN binding site of CD9 EC2 exhibited reduced FN-mediated haptotactic motility (Fig. 4A). Cells expressing CD9Δ152–192 demonstrated a 60% reduction in motility over 6 h compared with CHO cells expressing full-length CD9 (p < 0.05). CD9Δ133–192 exhibited a 64% reduction in motility (p < 0.05). CD9Δ113–228–CHO (deletion of CD9 EC2, TM4, and COOH-terminal) showed a 58% reduction in motility to FN. Differences in the values for percent reduction of motility for cells expressing CD9 EC2 deletions Δ113–228, Δ133–192, and Δ152–192 were not statistically significant. Cells transfected with a CD9 truncation where part of the FN binding site was expressed (CD9Δ173–192-CHO) exhibited a reduction in motility of 45%. Motility of CHO cells expressing CD9 EC1 deletion (Δ23) was compared with its mock transfection (Mock-Zeo), CD9-CHO-N3 (complete wild type CD9 sequence). Deletion of CD9 EC1 had no significant effect on CHO cell motility to FN (p > 0.05) (Fig. 4B).

To further characterize the region of CD9 EC2 responsible for CD9-modulated CHO cell haptotaxis, motility was assessed under conditions where FN-coated tissue culture inserts had been blocked with peptide 6 corresponding to CD9 EC2 aa 168–192. As shown in Fig. 4C, 45% inhibition of CD9-directed CHO cell motility to FN was observed in the presence of peptide 6. The specificity of this effect was demonstrated by use of a scrambled version of peptide 6 (peptide 6S). In summary, these results point to the importance of the FN-binding region on CD9 EC2 for cellular haptotactic motility and suggest that CD9 EC2 aa 168–192 contains a functional domain associated with FN-directed motility. The structures of CD9 truncations are shown in Fig. 5.

DISCUSSION

The TM4SF member CD9 has been implicated in several cellular functions, including motility, proliferation, and spreading. The mechanism of CD9 effects on these functions is not clear, but CD9 may act via direct interaction with ECM proteins or indirectly via the modulation of integrin-mediated signaling pathways. The direct interaction between CD9 and several ECM proteins was investigated. CD9 was found to specifically bind to FN in a Ca\(^{2+}\)-dependent manner. FN has recognition sequences that mediate cell attachment, cell spreading, and migration as well as pericellular FN matrix assembly. Thus, CD9 may elicit some of its effects by modulating cell-FN interactions as a result of CD9 protein binding directly to FN. Studies using CD9-transfected CHO-B2 cells demonstrated that CD9 is able to mediate CHO cell adhesion to FN in the absence of the classic FN receptor, integrin α\(_5β_1\). The identification of FN as a direct target of CD9 binding provides the first readily measured receptor function for CD9.

The validity and specificity of the CD9-FN interaction were demonstrated by complementary approaches: 1) FN was shown to bind to either purified platelet CD9 or a recombinant form of CD9, and 2) the FN binding site on CD9 was partially defined using synthetic CD9 peptides, one of which competitively inhibited CD9-FN interaction. The ELISA studies provided strong support for a specific interaction between CD9 and FN. To eliminate the possibility that the FN binding assessed by these methods may be due to contamination of an integrin in the purified CD9 preparation, we generated a bacterially expressed recombinant form of CD9 and demonstrated that both platelet-derived and bacterially generated CD9 had a similar affinity for FN binding. In support of these data, a specific association between FN captured by anti-FN mAbs and purified CD9 was detected by surface plasmone resonance biosensor analysis. This demonstration of CD9-FN interaction confirmed that CD9 was also able to bind to FN in its native conformation. FN bound directly to Peptide 6, which corresponds to CD9 residues 168–192, a portion of CD9 EC2. Peptide 6 partially inhibited the CD9-FN interaction in competition studies. These data suggest that CD9 residues 168–192 contain part, but not all, of the FN-binding sequences on CD9 EC2.

CD9- and Mock-CHO cells were generated to study the effects of CD9 expression on cell adhesive function. The CD9-CHO-A6 cells had increased spread morphology on FN in adhesion assays (13). Here, we demonstrate that CD9 expression significantly enhances FN-directed CHO-cell haptotactic motility, and residues 168–192 of CD9 EC2 are associated with increased FN-directed cell motility. Based on these findings we postulate that extracellular ligand interaction is required for CD9-mediated cell haptotactic motility.

The data described in this study clearly demonstrate that CD9 expression induces increased CHO cell motility. These findings are at variance with earlier studies showing that CD9 expression on CHO cells resulted in a reduction in CHO cell motility (20, 21). There are several possible reasons for these conflicting data. CHO-K1 was the parental cell line for our studies, in contrast to previous reports where mutant CHO cell lines that had specific nutritional requirements were used to generate CD9-expressing CHO cell lines (20, 21). Additionally, our study specifically focused on CD9 effects on CHO cell motility to FN. Earlier studies examined the effects on CD9 expression on the ability of CHO cells to penetrate Matrigel-coated filters. The Matrigel preparation described in these studies consisted of primarily laminin, collagen IV, and heparan sulfate proteoglycan and did not contain significant quantities of FN (22, 23). The cell invasion assays described in these studies were run for of 16–24 h as compared with the 3–6 h where statistically significant enhancement of CHO cell motility to FN was observed on CD9 expression. Previous reports have utilized phagokinetic track assays to study CD9 effects on CHO cell motility that provide readouts of random motility. In contrast, our studies specifically address CD9-haptotactic motility to a specific ligand, FN.

How CD9 influences cellular phenotype is not yet clear. The data presented here suggest that CD9 influences cell adhesive functions by binding directly to FN. Numerous studies have demonstrated that CD9 can form complexes with other adhesive molecules such as integrins (1). CD9 has been detected in a number of integrin complexes that include α\(_5β_1\), α\(_4β_1\), α\(_3β_1\), and α\(_3β_1\) (1, 24–26). In addition, CD9, CD81, and β\(_1\) subunits were detected in the cell membrane footprints and rippings of motile keratinocytes suggesting a role for tetraspanin-integrin complexes in adhesion to ECM and keratinocyte motility (27). It has also been proposed that CD9 modulates integrin-mediated signaling pathways. A recent study (28) has shown that a subset of TM4SF members, including CD9, can act as linker proteins for the recruitment of...
of protein kinase C to integrins. The specificity for protein kinase C association was thought to depend upon the cytoplasmic tails or the first two TM regions of the TM4SF members. A specific association between α5β1, the TM4SF member CD151, and phosphatidylinositol 4-kinase was detected in neurophilts, and antibodies to either α5β1 or CD151 were able to reduce neurophil motility (29). It is conceivable that CD9 and α5β1 work in concert, because the down-regulation of both has been associated with decreased metastatic potential (30). In support of this idea, CD9 was detected in complex with α5β1 in CD9-transfected CHO cell lysates.3

TM4SF EC2 domains appear to be critical for their associations with other proteins. For example, amino acids 186–217 of CD151 EC2 and aa 570–705 of the α5 subunit were required for CD151/α5β1 integrin association (24). Their unique EC regions confer different cell type- or stage-dependent functions. The coordination of CD9 direct ligand binding activity and its association with other surface molecules may be in part responsible for mediating cell-specific functions. In support of this idea, a recent study has shown that CD9 is involved in the invasion of a human choriocarcinoma cell line (32), and CD9 was detected in complex with α5 integrins on the surface of this cell line (27).

In summary, CD9 represents a new class of FN receptor. CD9 shows no structural homology to the integrin family of FN receptors (33). However, recent evidence suggests that CD9 may function by binding to FN and by associating with various members of the integrin family or other surface molecules (1, 24–26, 30). Because transmembrane regions of CD9 and the other tetraspanin proteins are highly conserved, it seems likely that the transmembrane regions are important in mediating common signaling events, whereas their unique EC regions confer different cell type- or stage-dependent functions. The coordination of CD9 direct ligand binding activity and its association with other surface molecules may be in part responsible for mediating cell-specific functions. In support of this idea, a recent study has shown that CD9 is involved in the invasion of a human choriocarcinoma cell line (32), and CD9 was detected in complex with α5 integrins on the surface of this cell line (27).

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