

Ectopic Expression of Human and Feline CD9 in a Human B Cell Line Confers β 1 Integrin-dependent Motility on Fibronectin and Laminin Substrates and Enhanced Tyrosine Phosphorylation*

(Received for publication, April 21, 1995, and in revised form, June 14, 1995)

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Few molecules have been shown to confer cell motility. Although the motility-arresting properties of anti-CD9 monoclonal antibody (mAb) suggest the transmembrane 4 superfamily (TM4SF) member CD9 can induce a motogenic signal, gene transfection studies have failed to confirm this hypothesis. We report here that ectopic expression of human CD9 (CD9h) and feline CD9 (CD9f) in the CD9-negative, poorly motile, human B cell line Raji dramatically enhances migration across fibronectin- and laminin-coated polycarbonate filters. Migration of Raji/CD9h and Raji/CD9f on either substrate was inhibited by the anti-CD9 mAb 50H.19 and by the anti- β 1 integrin mAb AP-138. Migration of Raji/CD9h on laminin was potently inhibited by the anti-VLA-6 integrin mAb GoH3 and by the anti-VLA-4 integrin mAb 44H6, whereas migration of Raji/CD9h on fibronectin was inhibited only by mAb 44H6. Since CD9h-transfected Raji cells adhered to fibronectin as effectively as mock transfectants, expression of CD9 enhanced motility, but not adhesion. CD9-enhanced migration was inhibited by the protein tyrosine kinase inhibitor herbimycin A suggesting that tyrosine phosphorylation played a role in the generation of a motogenic signal. Raji/CD9h transfectants adherent to fibronectin expressed 6-fold higher levels of phosphotyrosine than Raji. Raji/CD9f transfectants also phosphorylated proteins on tyrosine more effectively than Raji including a protein of 110 kDa which was phosphorylated on the motility-inducing substrates laminin and fibronectin, but not on bovine serum albumin. Our results support a role for CD9 in the amplification of a motogenic signal in B cells involving β 1 integrins and the activation of protein tyrosine kinases.

CD9, a 22–24-kDa cell surface glycoprotein, highly expressed in developing B cells, blood platelets, neuroblastoma cell lines, normal and transformed epithelia, peripheral glia, and neurons (1–4) is a member of the transmembrane 4 protein superfamily (TM4SF).¹ TM4SF proteins possess two external

loops and four hydrophobic domains of membrane-spanning length (5–8). The putative transmembrane regions and certain residues within the external loops are highly conserved suggesting the proteins perform closely related functions. However, the nature of those functions is not well understood (6). There is evidence that TM4SF proteins play a role in the initiation of signals controlling cell proliferation. For example, CD53 is found exclusively on subsets of proliferating thymocytes (9), CD81 exerts both positive and negative effects on the proliferation of T and B lymphoid cell lines (10), and an anti-CD9 mAb was recently shown to induce the proliferation of Schwann cells (11). Some family members may also regulate adhesive and morphogenetic functions. CD9 is expressed at high density on peripheral blood platelets (12), and anti-CD9 mAb are exceptional platelet agonists (2, 13) when Fc-receptor interactions are not impeded (14). Immobilized, but not soluble, F(ab')₂ fragments of anti-CD9 mAb activate platelets (15), and immobilized, but not soluble, antibody induces proliferation in nerve cells (11). These findings suggest that CD9 may transduce signals involving immobilized ligands such as extracellular matrix proteins. Integrins are heterodimeric adhesion molecules linking extracellular matrix proteins to an active cytoskeleton (16). We have reported that anti-CD9 mAb promote physical association between CD9 and the β 3 integrin GPIIb-IIIa (17), and that anti-CD9 mAb induce homotypic and heterotypic adhesive interactions in pre-B cells through pathways which may involve β 1 integrins (18, 19). Very recently, these observations have been extended by a report that CD9 physically associates with β 1 integrins (20). CD9 may therefore interact with integrins to participate in the transduction of integrin-dependent signals across the plasma membrane (17, 20).

Antibodies which uniquely prevent cell motility in a variety of tumor cell lines were found to recognize a protein subsequently identified as CD9 suggesting that CD9 is a positive regulator of cell motility (14). However, ectopic expression of CD9 in heterologous tumor cell lines either failed to confer motility (20, 21) or in some cases suppressed migratory and metastatic activity (21). We chose to study CD9 function in cells of the B lymphocyte lineage in which CD9 expression is modulated in a stage-specific manner. Pre-B lymphocytes express high levels of CD9, and we have observed that transformed pre-B cell lines readily penetrate fibronectin-coated polycarbonate filters. B cell lines on the other hand lack CD9 and are

* This work was supported in part by an operating award from the Medical Research Council of Canada (to A. R. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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^{††} Supported by the Wellcome Trust.

¹ The abbreviations used are: TM4SF, transmembrane 4 superfamily; VLA, very late antigen or β 1 integrins; RHAMM, receptor for hyalu-

ronic acid-mediated motility; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter.

poorly migratory in Transwell assays (21). To investigate the possibility that CD9 plays a role in integrin-dependent motility, we transfected cDNA encoding human and feline CD9 into the immature B cell Raji which lacks CD9. Raji expresses the $\beta 1$ integrin VLA-4, a fibronectin receptor found in highly motile cells (22–26) implicated in invasiveness, and metastasis (22, 23), as well as the laminin receptor VLA-6. In our experiments, Raji cells penetrated laminin or fibronectin-coated filters poorly, suggesting they might lack an accessory molecule required for migration on integrin-dependent substrates. We report here that ectopic expression of CD9 in Raji cells exposed to laminin and fibronectin dramatically enhanced both their ability to penetrate polycarbonate filters and their ability to phosphorylate protein targets on tyrosine.

EXPERIMENTAL PROCEDURES

Molecular Cloning of CD9—A full-length cDNA encoding CD9 was selected using the anti-CD9 mAb 50H.19 from an endothelial cDNA library assembled in the expression vector λ gt11 (Clontech) and the 1.2-kilobase insert subcloned into PTZ-19, transformed into XL1-Blue, and single-stranded template DNA isolated for sequencing. The clone contains the entire coding region for CD9 flanked by 51-base pair 5'- and 450 base pair 3'-untranslated regions and is identical with the published coding sequence (8). The insert was cloned into the eukaryotic Epstein Barr virus episomal plasmid expression vector pREP4 (Invitrogen), transfected into Raji by electroporation, and selected by hygromycin resistance, followed by immunoselection on immobilized mAb 50H.19. Mock-transfected controls containing the vector alone were selected on the basis of hygromycin-resistance. A full length cDNA encoding feline CD9 was obtained using the rapid amplification cDNA ends technique as described (24), cloned into the cDNA3 expression vector, and transfected into the same strain of Raji by electroporation, followed by immunoselection with anti-CD9 mAb.

Cell Lines, mAb, and FACS Analysis—HOON and Raji are pre-B cell and B cell lines, respectively. They were obtained from Dr. Michelle Letarte (University of Toronto) and Dr. B. M. Longenecker (University of Alberta) and maintained in RPMI 1640, 10% fetal calf serum. For FACS analysis cells were stained with mAb TS2/7 against VLA-1 (T Cell Diagnostics, Cambridge, MA), mAb P1E6 against VLA-2, (Life Technologies, Inc.), mAb P1B5 against VLA-3 (Life Technologies, Inc.), mAb 44H6 against VLA-4 (kindly provided by Dr. Michelle Letarte), mAb P1D6 against VLA-5 (Life Technologies, Inc.), and mAb GoH3 against VLA-6 (AMAC, BioCan, Mississauga, Ontario) using a FACS-can cell sorter (Becton Dickinson) as described previously (18). mAb

50H.19 is an anti-CD9 mAb first raised against a melanoma cell line (25). mAb 4B4 (Coulter Corp., Miami, FL) and mAb AP-138 recognize the $\beta 1$ integrin subunit. Isotype-matched controls were included, and >10,000 events accumulated per sample. Data were analyzed using the Lysis II program.

Immunoprecipitation and Immunoblotting— 10^6 HOON cells were lysed in 0.5 ml of RIPA buffer, precleared with Protein A-Sepharose, and immunoprecipitated by addition of 5 μ g of the anti-CD9 mAb 50H.19 together with 30 μ l of preswollen Protein A-Sepharose or Protein A-Sepharose alone, and eluted with SDS-PAGE loading buffer. Following separation on a 5–20% gradient gel, the proteins were transferred to nitrocellulose and immunoblotted with horseradish peroxidase-conjugated mAb 50H.19. Positive bands were detected by enhanced chemiluminescence (Amersham International, Buckinghamshire, United Kingdom).

Adhesion Assay—Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and washed in RPMI containing 0.5% BSA. 2×10^6 cells in a volume of 250 μ l of RPMI 1640, 0.5% BSA were added to a 24-well plate in which wells were precoated for 2 h with varying concentrations of human plasma fibronectin (Life Technologies, Inc.) in phosphate-buffered saline at 37° C and blocked for 1 h with 1% BSA (26). Control wells were incubated with BSA alone. The effect of inhibitors was determined by preincubation with the agent for 20 min at 23° C and addition to the assay without further dilution. Cells were

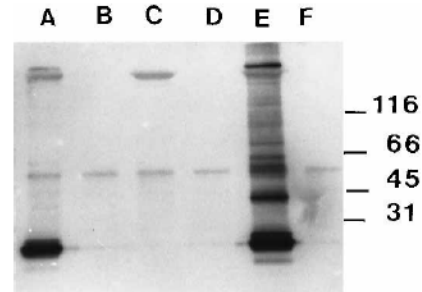


FIG. 1. Ectopic expression of CD9 in Raji. Cell lysates from the CD9+ pre-B cell line HOON (lanes A and B), the CD9- B cell line Raji (lanes C and D), and Raji transfected with a full-length cDNA encoding human CD9 (lanes E and F) were immunoprecipitated with the anti-CD9 mAb 50H.19/Protein A-Sepharose (lanes A, C, and E) or with Protein A-Sepharose alone (lanes B, D, and F). Following SDS-PAGE, the proteins were immunoblotted with horseradish peroxidase-conjugated mAb 50H.19. Molecular size markers: 116 kDa, 66 kDa, 45 kDa, and 31 kDa.

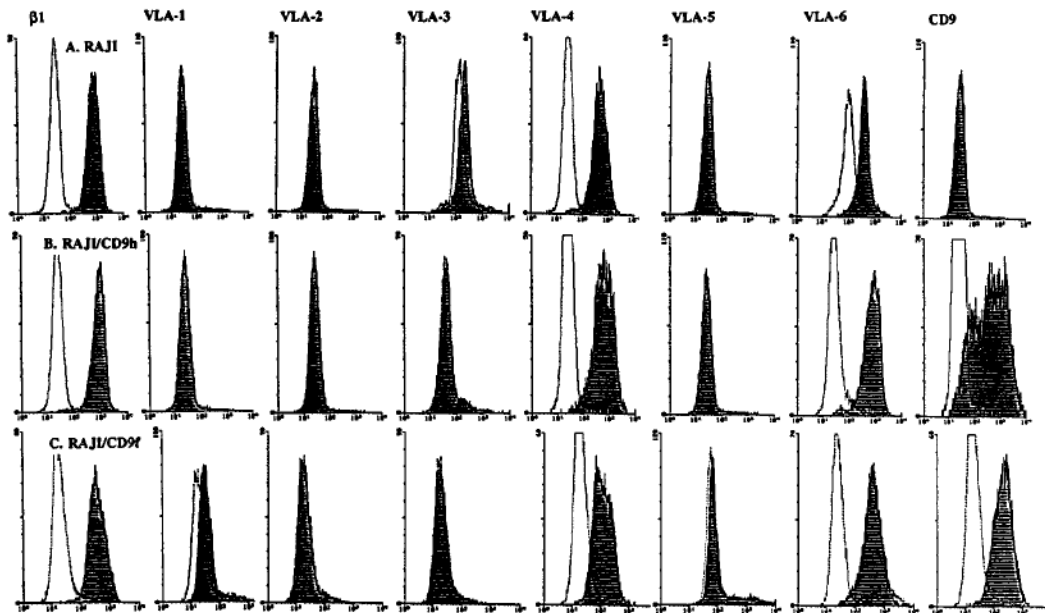
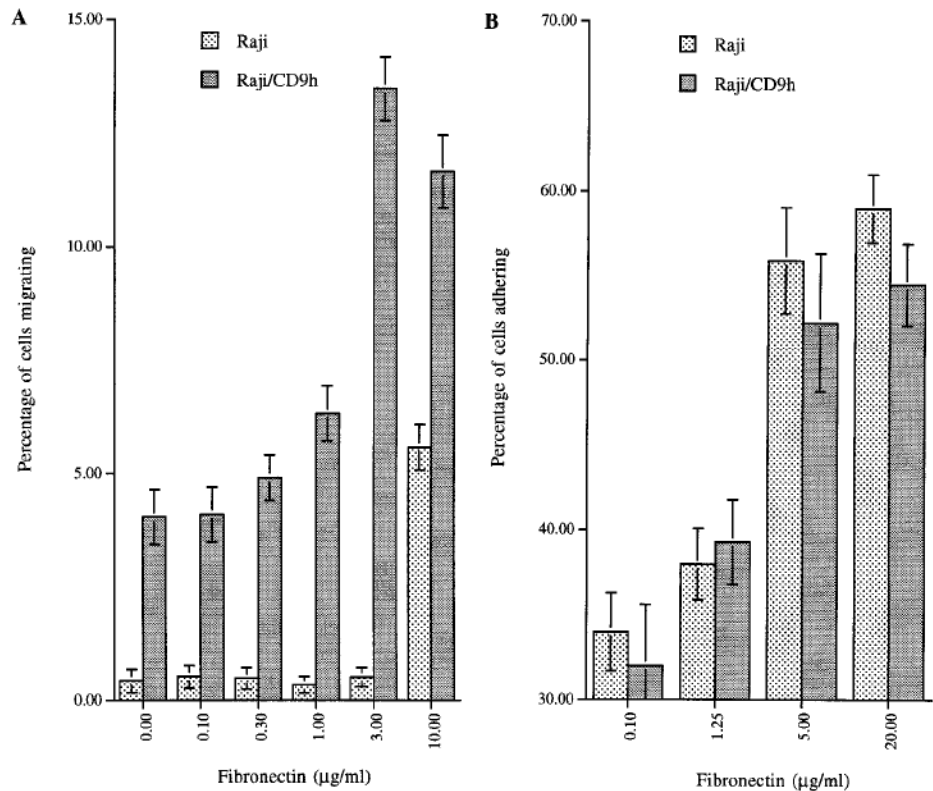


FIG. 2. FACS analysis of VLA antigen and CD9 expression on Raji, Raji transfected with human CD9 (Raji/CD9h), and Raji transfected with feline CD9 (Raji/CD9f). Cells were stained with mAb against VLA antigens and CD9 as described under "Experimental Procedures" followed by a fluorescein isothiocyanate-conjugated second antibody and analyzed on a FACSscan. The fluorescence profiles of stained cells (black) are compared to isotype controls (white).

FIG. 3. *A*, motility of Raji and of Raji/CD9 transfectants on fibronectin-coated filters. Motility was assessed by counting the number of cells penetrating the lower chamber of a Transwell apparatus through a perforated polycarbonate filter (8- μ diameter pore). The *abscissa* shows the coating concentration of plasma fibronectin. Error bars = 1 S.D. The result is representative of six experiments. *B*, adhesion of Raji and Raji/CD9 to fibronectin-coated plates. Cells were plated on wells coated with varying amounts of plasma fibronectin, and adhesion-quantitated after 90 min by mechanically agitating the plate and counting the nonadherent cells. The *abscissa* shows the coating concentration of fibronectin. The result is representative of five experiments. Error bars = 1 S.D.



preincubated at 37° C for 90 min before dislodging the loosely adherent cells by agitation on a rotary shaker at 110 rpm for 5 min. Detached cells were quantitated in an automated cell counter (Coulter Electronics, HiLeah, FL).

Motility Assay—Cells were washed and resuspended in RPMI 1640, 0.5% BSA. 1.5×10^5 cells in a volume of 100 μ l were applied to the upper chamber of 6.5-mm diameter Transwells (Costar, Sin-Can Inc., Calgary, Alberta), and 600 μ l of RPMI 1640, 0.5% BSA were added to the lower chamber. Polycarbonate filters (8- μ diameter pores) were precoated with 100 μ l of protein solution for 2 h and blocked with 1% BSA (26). Cells were incubated for 18 h at 37° C, and cells migrating to the lower chamber were quantitated in an automated cell counter (Coulter Electronics). Examination of the lower surface of the filter confirmed that transmigrating cells did not adhere to the filter, but accumulated in the lower chamber.

Analysis of Phosphotyrosine—250- μ l aliquots of Raji or Raji/CD9 transfectants at concentrations of 5×10^5 to 8×10^6 cells/ml were introduced into the wells of a 24-well tissue culture plate (Costar) precoated with fibronectin and blocked with BSA. Cells were incubated for 90 min at 37° C and then lysed in RIPA buffer (pH 8.0) containing 0.02 M Tris-HCl, 0.001 M Na_2HPO_4 , 137 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate; 1 μ g/ml leupeptin, pepstatin A, antipain, and Trasylol; 1 mM EGTA; 1 mM iodoacetamide; 1 mM sodium orthovanadate. Proteins were separated by 5–20% SDS-PAGE, transferred to nitrocellulose, immunoblotted with the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (Transduction Laboratories, Lexington, KY), and developed using enhanced chemiluminescence.

RESULTS

Ectopic Expression of CD9 in Raji—CD9 was strongly expressed in lysates of Raji cells transfected with the human CD9 cDNA insert (Raji/CD9h), but not in Raji transfected with pREP4 alone (Fig. 1, lanes E and C). The level of expression was very similar to that of the highly motile pre-B cell line HOON (Fig. 1, lane A). FACS analysis demonstrated that our strain of Raji cells expressed predominantly VLA-4 and VLA-6 among β 1 integrins, and that ectopic expression of CD9 did not qualitatively affect their VLA profile (Fig. 2). However, ectopic expression of both human and feline CD9 markedly increased the expression of VLA-6 (Fig. 2) suggesting that CD9 may

TABLE I

Effect of Inhibitors on cell migration

Raji/CD9 or HOON cells were preincubated with 10 μ g/ml mAb 50H.19, AP-138, MOPC 21, or the protein tyrosine kinase inhibitor herbimycin A, and the percentage of cells migrating through an 8- μ pore diameter polycarbonate filter coated with 3 μ g/ml fibronectin was assayed over a period of 18 h.

Agent	% inhibition of migration \pm S.D.	
	Raji/CD9h	HOON
MOPC 21 (IgG2a control)	0.5 \pm 4.6	ND ^a
mAb 50H.19 (anti-CD9)	70.4 \pm 8.2	71.2 \pm 5.7
mAb AP-138 (anti- β 1)	59.0 \pm 9.1	72.4 \pm 3.1
Herbimycin A (2.6 μ M)	79.5 \pm 6.3	ND ^a

^a ND, not determined.

preferentially affect the transport or assembly of this integrin.

CD9 Enhances Motility of Raji on Fibronectin—When Raji and Raji/CD9 transfectants were compared for their ability to transmigrate across fibronectin-coated polycarbonate filters, the transfectants exhibited a dramatically enhanced migratory capacity (Fig. 3A). Migration of the transfectants increased in proportion to the coating concentration of fibronectin, reaching a maximum at 3 μ g/ml. In contrast, mock-transfected Raji cells barely migrated except at the highest coating concentration of 10 μ g/ml. Since Raji has the capacity to migrate on fibronectin, but requires a considerably higher concentration to become motile, it suggests that CD9 expression may amplify a motorogenic signal induced by fibronectin. Tyrosine kinases have recently been implicated in cell motility (27, 28). We therefore asked whether preincubation of the cells with the protein tyrosine kinase inhibitor herbimycin A would affect cell migration. Incubation of cells for 3.5 h with herbimycin A reduced tyrosine phosphorylation by >70% without affecting cell viability. Preincubation of Raji/CD9 transfectants with herbimycin A for this period inhibited migration by almost 80% (Table I), indicating that tyrosine kinase activity was required for CD9-enhanced cell migration.

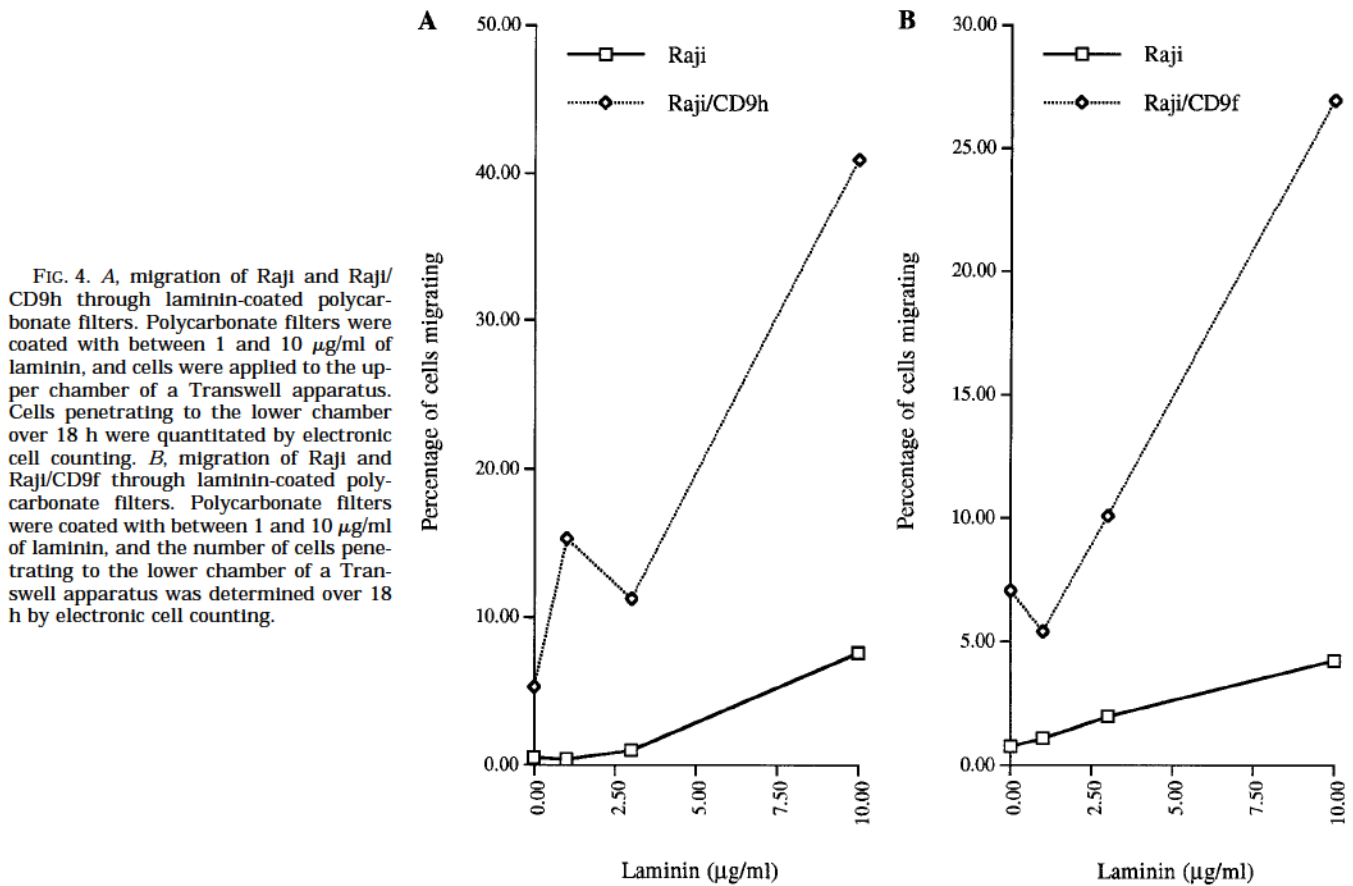


FIG. 4. *A*, migration of Raji and Raji/CD9h through laminin-coated polycarbonate filters. Polycarbonate filters were coated with between 1 and 10 $\mu\text{g/ml}$ of laminin, and cells were applied to the upper chamber of a Transwell apparatus. Cells penetrating to the lower chamber over 18 h were quantitated by electronic cell counting. *B*, migration of Raji and Raji/CD9f through laminin-coated polycarbonate filters. Polycarbonate filters were coated with between 1 and 10 $\mu\text{g/ml}$ of laminin, and the number of cells penetrating to the lower chamber of a Transwell apparatus was determined over 18 h by electronic cell counting.

Since CD9 is reported to associate with $\beta 1$ integrins (20), we investigated whether $\beta 1$ integrins were involved in cell migration on fibronectin by preincubating the cells with mAb AP-138, an antibody which recognizes the $\beta 1$ integrin subunit. mAb AP-138 inhibited cell motility by 59% indicating that $\beta 1$ integrins play a regulatory role in the migratory behavior (Table I). In keeping with reports that anti-CD9 mAb block cell movement in a variety of transformed cells (20, 21, 29), we observed that the anti-CD9 mAb 50H.19 inhibited migration of the transfectants by 70.4% (Table I). mAb 50H.19 and mAb AP-138 also blocked motility of the highly motile pre-B cell line HOON by 71.2 and 72.4%, respectively. Transfection of CD9 therefore confers motility upon a poorly motile B cell line which is inhibitable by anti-CD9 mAb in the manner of pre-B cells which constitutively express the protein.

CD9 Does Not Confer Enhanced Adhesion to Fibronectin—B cells exposed to surfaces coated with fibronectin adhere weakly in comparison to pre-B cells (30). To investigate whether CD9 influenced the avidity of cellular adhesion of B lymphocytes, we quantitated binding of Raji and Raji/CD9 to surfaces coated with fibronectin. Because B lymphoid cells possess relatively low avidity for most extracellular matrix proteins, we developed a low stringency adhesion assay in which the usual method of detaching loosely adherent cells by washing was replaced by a 10-min period of mechanical agitation. Using this assay, we found that both Raji and Raji/CD9 transfectants adhered to fibronectin in a dose-dependent manner, and that the CD9 transfectants adhered similarly to mock-transfected controls (Fig. 3B). Since ectopic expression of CD9 did not significantly affect adhesion of the B cell line CD9 does not appear to influence motility by increasing the level of cellular avidity for fibronectin or by inducing a generalized state of cellular activation. Preincubation of the cells with herbimycin A did not affect adhesion, indicating that adhesion unlike CD9-

enhanced motility does not require tyrosine phosphorylation (results not shown).

CD9-transfected Raji Exhibit Enhanced Migration on Laminin—Although Raji possesses VLA-6, a laminin receptor, it is poorly motile on laminin. We asked whether cells transfected with CD9 would show enhanced motility on laminin. Raji/CD9h transfectants demonstrated a dramatic increase in motility over Raji controls on polycarbonate filters coated with laminin which increased with the coating concentration between 1 and 10 $\mu\text{g/ml}$ (Fig. 4A). Raji transfected with feline CD9 also exhibited a laminin-dependent increase in migration over mock-transfected controls (Fig. 4B). The ability of CD9 from two different species to confer large increases in cell motility on laminin substrates strongly suggests that motility enhancement is a fundamental property of CD9. In a second experiment, Raji/CD9f was found to show enhanced migration on both fibronectin and laminin, but not BSA (Fig. 5), confirming that CD9 confers motility on substrates recognized by the two major Raji VLA-antigens.

CD9 Enhanced Migration Is Inhibitable by Antibodies against VLA-4 and VLA-6—The ability of anti- $\beta 1$ integrin subunit mAb to inhibit the migration of Raji/CD9h on extracellular matrix implicates VLA in the generation of a motogenic signal. To investigate which VLA is responsible for the inhibition, cells were preincubated with the anti-VLA-4 mAb 44H6 and with the anti-VLA-6 mAb GoH3. Both antibodies strongly inhibited migration of Raji/CD9h on laminin, and their combination completely prevented cells entering the lower chamber (Fig. 6A), whereas mAb 44H6, but not mAb GoH3, significantly inhibited migration on fibronectin-coated filters (Fig. 6B). Since VLA-6 selectively regulates migration on laminin, but cross-linking VLA-4 affects migration on either laminin or fibronectin, it suggests that VLA-4 plays an unexpected and essential role in B cell motility. Cells transfected with feline

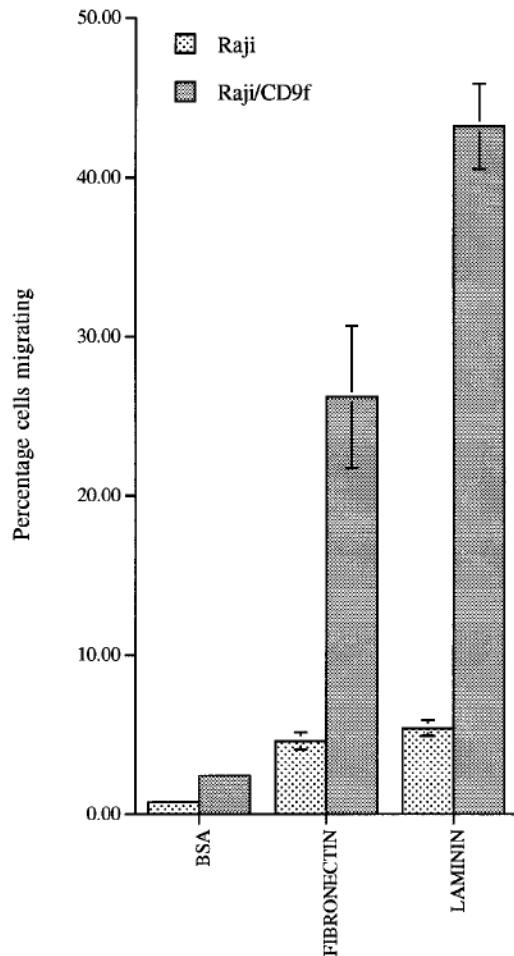


FIG. 5. Migration of Raji/CD9f through polycarbonate filters. Polycarbonate filters were coated with 10 μ g/ml plasma fibronectin, or laminin, and the percentage of cells penetrating to the lower chamber of a Transwell apparatus was determined over 18 h by electronic cell counting. Error bars represent 1 S.D. about the mean.

CD9 were similarly inhibited by both mAbs on laminin substrates, but the anti-VLA-6 mAb effectively inhibited migration on fibronectin implying a possible species difference (Fig. 6, C and D). The epitope recognized by the anti-VLA-4 mAb may be critical since the anti-VLA4 mAb P4G9 had no effect upon migration (results not shown). mAb against CD9 and the β 1 integrin subunit also effectively inhibited migration of both CD9h and CD9f transfectants on either substrate (Fig. 6, A, B, C, and D). These results implicate CD9 and the β 1 integrin subunit as major determinants of cell motility on laminin substrates through interactions involving VLA-4 and VLA-6.

The Migratory Subset Is Enriched for CD9 Expression—If CD9 is directly responsible for migratory activity, we reasoned that CD9 might be preferentially expressed in cells migrating to the lower chamber of the Transwell apparatus during the 18-h assay. FACS analysis of this subpopulation produced a mean fluorescence value for CD9 of 1386.5 compared to 854.3 for the starting cell population, an enrichment of 162%. In contrast, the mean fluorescence for β 1 integrin expression was essentially unchanged (1016.47 versus 959.71). Therefore, changes in CD9 expression rather than changes in β 1 expression correlate with the migratory behavior of the transfected cells. CD9 expression has been associated with both enhancement and suppression of cell proliferation (21, 29, 31). However, in our hands, the proliferation of cultures of CD9-transfected and mock-transfected Raji cells in log phase measured over a 4-day interval were identical (results not shown), indi-

cating that the effects of CD9 expression on motile behavior did not result from differential proliferative capacity.

Raji/CD9 Transfectants Display Increased Tyrosine Phosphorylation—Adherence to fibronectin or cross-linking of the β 1 integrin subunit with mAb is reported to activate protein tyrosine kinases in B cells and to phosphorylate proteins of 105–110 kDa (32). Since CD9 associates with β 1 integrins and enhances migration on fibronectin, we wondered whether CD9 could affect the ability of cells to phosphorylate proteins on tyrosine. We therefore investigated whether tyrosine phosphorylation accompanied contact of Raji or of Raji/CD9 transfectants with fibronectin substrates (Fig. 7). Tyrosine phosphorylation of several components was observed in Raji cells adhering to fibronectin, the major bands migrating at 69 and 130 kDa. Phosphorylation was observable by 5 min, and the intensity of the signal was observed to increase progressively over a 90-min period. The pattern of phosphorylation was identical in both transfected and mock-transfected cells, indicating that CD9 expression does not influence the specificity of response. However, CD9 transfectants consistently displayed enhanced levels of tyrosine phosphorylation. The optical density of the 130-kDa phosphotyrosine band derived from cells at a concentration of 4×10^6 ml was $5.9 \times$ greater for Raji/CD9 cells than for Raji, and the 69-kDa band was $6.2 \times$ greater for Raji/CD9 cells than for Raji. Pretreatment of Raji or of the CD9 transfectants with the tyrosine phosphatase inhibitor sodium orthovanadate enhanced the level of tyrosine phosphorylation in both transfected and mock-transfected cells, demonstrating that the activation of protein tyrosine kinases by immobilized fibronectin is strongly opposed by tyrosine phosphatase activity (Fig. 7). The enhanced phosphorylation observed in CD9-positive cells could therefore reflect a change in tyrosine kinase/phosphatase balance rather than an increase in overall kinase activity. Whether enhanced phosphorylation on tyrosine occurred in cells transfected with feline CD9 was determined by allowing cells to attach to BSA-, laminin-, and fibronectin-coated surfaces, carefully removing the medium, and lysing the cells. Cells adherent to all three surfaces strongly phosphorylated a protein of 72 kDa not seen in mock transfectants, whereas a band of 110 kDa was specifically phosphorylated on the motility-inducing substrates laminin and fibronectin, but not on cells exposed to BSA (Fig. 8). Consequently, enhanced phosphotyrosine signaling was observed in CD9-transfected cells adhering to a variety of substrates and a 110-kDa band specifically observed on targets of the major B cell integrins. While additional experiments are required to clarify these issues, the results clearly indicate that CD9-transfected B cells have a markedly enhanced capacity for tyrosine phosphorylation on integrin-dependent substrates and may therefore play a role in the regulation of contact signaling.

DISCUSSION

Cell motility is a complex process involving extracellular matrix proteins, adhesion receptors, cytoskeletal components, and signaling molecules. A number of agents have been identified which modulate motility including fibronectin and certain growth factors, but few transmembrane molecules other than integrins have been shown to play key roles (33). CD9 (29) and the hyaluronan receptor RHAMM (34) are two non-integrin cell surface proteins demonstrated to affect motility. However, although CD9 was first identified through exhaustive selection of mAbs which block motile function, experimental investigation of CD9 function by gene transfection has not supported a role for CD9 as a motogenic molecule. For example, transfection of CD9 into the CD9-positive human lung adenocarcinoma cell line MAC10 led to a reduction rather than gain in cellular motility (21). This might be explained by a gene

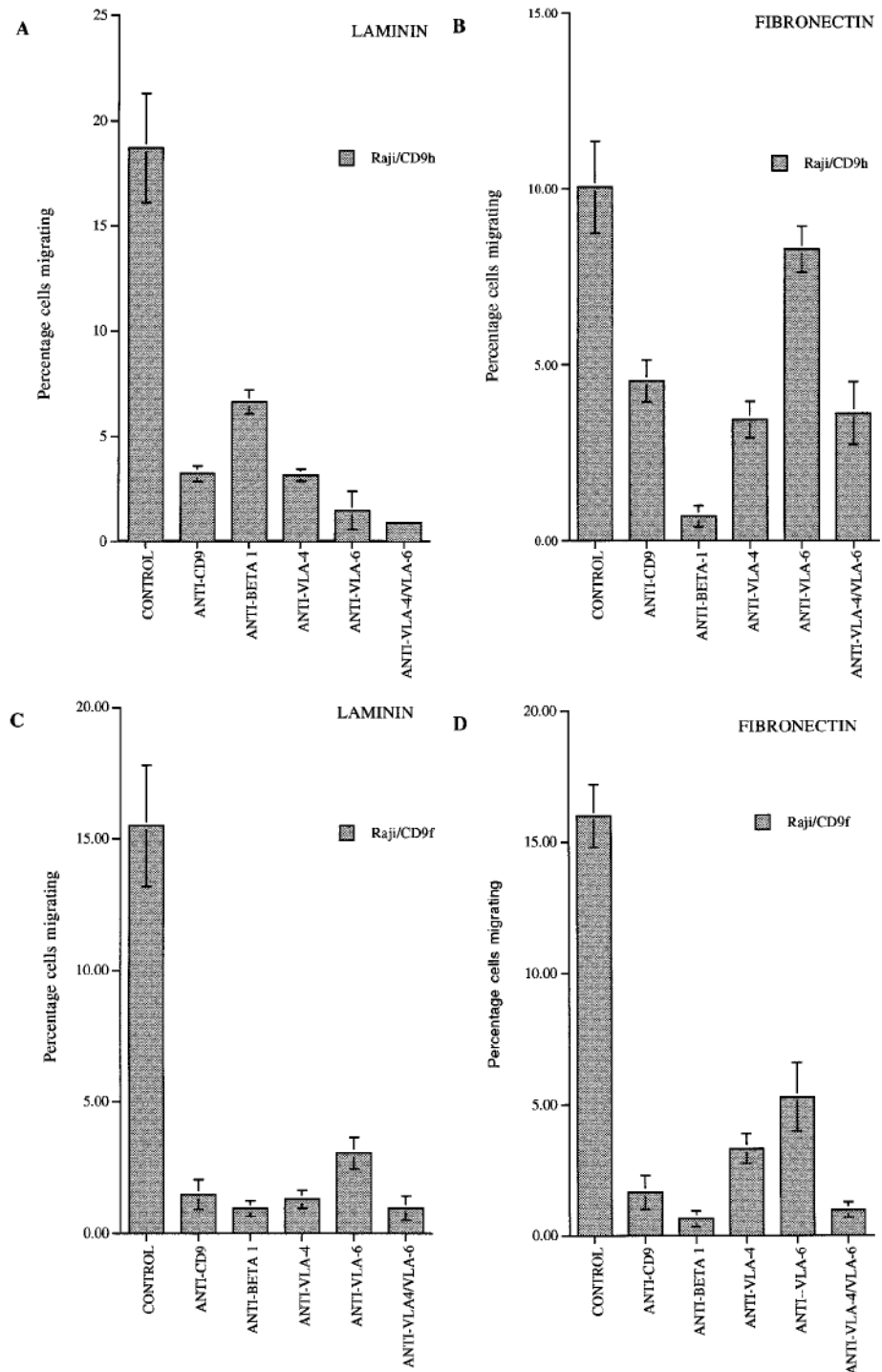


FIG. 6. Effect of antibodies to VLA-antigens and CD9 on the migration of Raji/CD9h and Raji/CD9f through laminin- or fibronectin-coated polycarbonate filters. Cells were preincubated with the anti-CD9 mAb 50H.19, the anti- β 1 subunit mAb AP-138, the anti-VLA-4 mAb 44H6, the anti-VLA-6 mAb GoH3, or a combination of mAb 44H6 and GoH3 at 10 μ g/ml for 30 min at 37°C before introduction into the upper chamber of a Transwell apparatus. Cells penetrating to the lower chamber were quantitated over 18 h. *A*, Raji/CD9h on laminin-coated filters. *B*, Raji/CD9h on fibronectin-coated filters. *C*, Raji/CD9f on laminin-coated filters. *D*, Raji/CD9f on fibronectin-coated filters. Laminin and fibronectin were coated at a concentration of 10 μ g/ml.

dosage effect in which overexpression of CD9 is inhibitory. However, overexpression cannot explain why transfection of CD9 into the CD9-negative human myeloma cell line ARH77 also suppressed motility (21), or why transfection of CD9 into the T cell line CEM failed to influence motility at all (20). Furthermore, the conclusion that CD9 is a suppressor of cell motility and metastasis (21) seems at odds with its widespread expression in carcinoma cell lines and biopsy specimens (29). The evidence to date therefore suggests a complex role for CD9 which may be critically affected by the cellular environment in which CD9 is expressed. Since CD9 is expressed at early and late stages of B cell differentiation, but not in nonactivated B cells, we chose to express CD9 in Raji cells which possess a

phenotype typical of an early B lymphocyte.

Our findings partially redress the paradox of CD9 function by demonstrating that Raji cells expressing physiological levels of CD9 and β 1 integrins provide a permissive environment for CD9-dependent motility on extracellular matrix proteins which can be blocked by antibodies against CD9 and the two major integrins. The finding that both human and feline CD9 effect very similar increases in motility involving β 1 integrins confirms that CD9 modulates migratory responses to extracellular matrix proteins and is consonant with the 95.1% homology in amino acid sequence between the two proteins (24). However, the effectiveness of anti-VLA-6 mAb in inhibiting the motile response of Raji/CD9f, but not of Raji/CD9h, on fibronectin

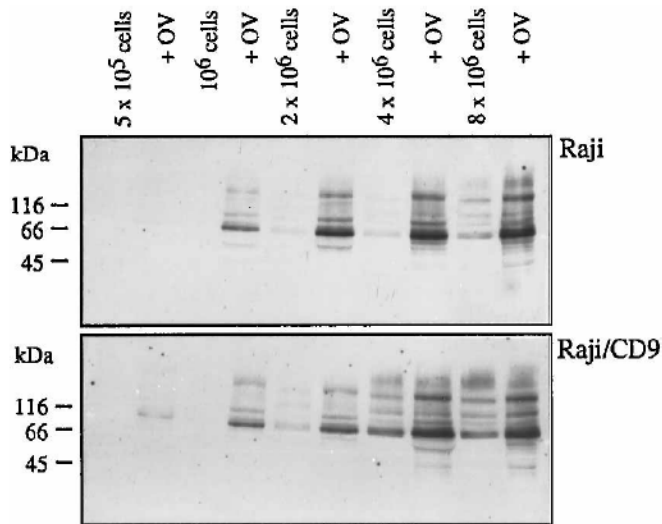


FIG. 7. Fibronectin-stimulated tyrosine phosphorylation of Raji and Raji/CD9. Graded numbers of cells were allowed to attach to fibronectin-coated surfaces ($5 \mu\text{g/ml}$ plasma fibronectin) for 90 min in the presence or absence of sodium orthovanadate (OV), lysed in RIPA buffer, and the proteins were separated by SDS-PAGE and immunoblotted with horseradish peroxidase-conjugated anti-phosphotyrosine antibody. Densitometric comparison of phosphotyrosine bands obtained from 4×10^5 cells gave a ratio of Raji:Raji/CD9 of 1:5.9 (130 kDa) and 1:6.2 (69 kDa), respectively. The result is representative of five experiments.

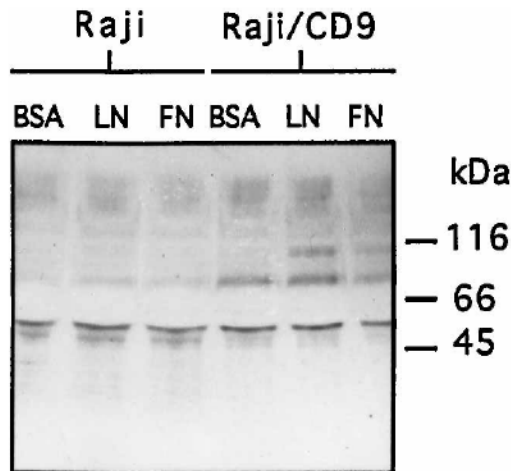


FIG. 8. Induction of phosphotyrosine in Raji and Raji/CD9f by exposure to fibronectin, laminin, and BSA. 4×10^5 cells were allowed to adhere to plastic surfaces coated with BSA, laminin (LN), and fibronectin (FN) at $10 \mu\text{g/ml}$ for 15 h before lysis in RIPA buffer, analysis by SDS-PAGE, and immunoblotting with horseradish peroxidase-conjugated anti-phosphotyrosine mAb.

substrates suggests a regulatory role for those regions of CD9 which are differentially expressed including amino acids 169–180 of the second external loop and a potential *N*-linked glycosylation site within the first external loop (24). Our finding that an anti-VLA-4 mAb inhibited migration of the transfectants on laminin to which VLA-4 is not known to bind was unexpected. Although VLA-4 is recognized to play a major role in the migratory activity of several cells, VLA-6 has been not been linked to cell motility, but rather to the regulation of differentiated events (35, 36). However, a recent report that an anti-VLA-6 mAb could partially inhibit pre-B cell transmigration under human bone marrow stroma suggests that VLA-6 may also function in cooperation with other adhesion molecules to modulate motile behavior (37). Possibly in our experiment, VLA-4 affects VLA-6-dependent motility through lateral interactions

between VLA-6 and sites of VLA-4 cytoskeletal association.

The presence of enhanced levels of tyrosine-phosphorylated proteins in CD9-transfected cells and the sensitivity of CD9-enhanced motility to inhibitors of tyrosine kinases suggest a connection between tyrosine phosphorylation and motility. Platelet-derived growth factor (38), epidermal growth factor (27, 39), and hepatocyte growth factor (40) have all recently been shown to induce cell motility through stimulation of their respective receptor protein tyrosine kinases. Integrin-dependent motility may be targeted by growth factors since it was recently reported that epidermal growth factor could induce motility through the selective modulation of events under the control of the integrin vitronectin receptor $\alpha\text{v}\beta\text{5}$ (28). In that study, the effect of epidermal growth factor was specific for motility, but had no effect upon cellular adhesion. Similarly, in the present study, CD9 modulated motility, but not adhesion, to fibronectin. In order to migrate, cells need to re-organize their actin networks through local assembly and deassembly of actin. Actin is assembled at points of substrate adherence known as focal contacts which also serve as sites of integrin localization. Recently it was reported that the hyaluronan receptor RHAMM promotes motility at least in part through tyrosine phosphorylation of the β1 integrin target, the focal adhesion kinase $\text{pp125}^{\text{FAK}}$ (41). CD9, through its ability to physically associate with β1 integrins, could therefore participate in either of these mechanisms. There is evidence that lymphocytes may transduce integrin-dependent signals through phosphorylation of proteins other than the focal adhesion kinase $\text{pp125}^{\text{FAK}}$. For example, stimulation of B lineage cells by fibronectin or by specifically cross-linking VLA-4 is reported to phosphorylate a protein of 105–110 kDa on tyrosine (32). The specific phosphorylation of a protein of this size in feline CD9 transfectants exposed to the motogenic substrates laminin and fibronectin, but not to BSA, could possibly implicate CD9 as an accessory component of VLA-4 signaling.

B cell development is accompanied by pronounced stage-specific changes in fibronectin receptor expression and cellular avidity, suggesting that interactions with fibronectin are of prime importance to B cell maturation (30, 42). We provide evidence that CD9, a putative β1 integrin accessory molecule expressed during early, but not late, B cell development, dramatically amplifies tyrosine phosphorylation and motility on fibronectin and laminin on reconstitution in a B cell. Such interactions are likely to be relevant to pre-B cell exploitation of inductive microenvironments within the bone marrow stroma and to the ability of leukemic cells and solid tumor cells to gain the motility required to disseminate to distant sites. Our results extend previous work indicating a physical association between CD9 and members of the β1 integrin family (17, 20) by suggesting that such associations may serve to amplify the ability of β1 integrins to activate protein tyrosine kinase-dependent signal pathways regulating motile, but not adhesive, behavior.

Acknowledgment—We are indebted to Helena Marusyk for her expert assistance with the photography.

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Ectopic Expression of Human and Feline CD9 in a Human B Cell Line Confers β 1 Integrin-dependent Motility on Fibronectin and Laminin Substrates and Enhanced Tyrosine Phosphorylation

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J. Biol. Chem. 1995, 270:24092-24099.
doi: 10.1074/jbc.270.41.24092

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