Mechanisms via c-Myc-Dependent and -Independent Very Late Antigen-4 Integrin and VCAM-1: A Novel Role for H-Ras in the Regulation of

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A Novel Role for H-Ras in the Regulation of Very Late Antigen-4 Integrin and VCAM-1 Via c-Myc-Dependent and -Independent Mechanisms

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Despite extensive studies on the crucial functions of Ras and c-Myc in cellular proliferation and transformation, their roles in regulating cell adhesion are not yet fully understood. Involvement of Ras in modulating integrin activity by inside-out signaling has been recently reported. However, in contrast to R-Ras, H-Ras was found to exhibit a suppressive effect. Here we show that ectopic expression of a constitutively active H-RasV12, but not c-Myc alone, in a hemopoietic cell line induces activation of very late Ag-4 (VLA-4, α4β1) integrin without changing its surface expression. Intriguingly, coexpression of H-RasV12 and c-Myc in these cells results in not only the activation of VLA-4, but also the induction of expression of VCAM-1, the counterreceptor for VLA-4, thereby mediating a marked homotypic cell aggregation. In addition, H-RasV12-induced VLA-4 activation appears to be partly down-regulated by coexpression with c-Myc. Our results represent an unprecedented example demonstrating a novel role for H-RasV12 in the regulation of cell adhesion via c-Myc-independent and -dependent mechanisms. The Journal of Immunology, 1999, 163: 4901–4908.

Cell adhesive interactions play crucial roles in directing the migration, proliferation, survival, and differentiation of cells (1). Integrins are the major family of cell-surface receptors that mediate cell adhesion and link extracellular ligands with cytoskeletal proteins (2). Integrins exhibit an aβ heterodimeric structure and have been divided into three major subgroups according to the β subunit expression: β1, β2, and β3. Within the β1 subfamily, also termed very late Ag (VLA)3 integrins, VLA-4 is unique in that it mediates both cell-to-cell and cell-to-extracellular matrix interactions by binding to its counterreceptor VCAM-1 and fibronectin (Fn), respectively (3). In contrast, VCAM-1 is an Ig-superfamily protein that is expressed on endothelial cells in response to IL-1α, IL-4, TNF-α, or LPS and is also expressed constitutively on a few other cell types, such as follicular dendritic cells in lymph nodes (4) and bone marrow stromal cells (5).

The binding activity of both integrins and VCAM-1 depends upon their surface expression, yet integrin activity can also be modulated through inside-out signaling (6). Although the precise molecular mechanism of integrin activation modulated by inside-out signaling remains largely unknown, multiple intracellular signaling pathways and proteins, such as protein kinase C (PKC) (7, 8), phosphoinositide 3-kinase (9, 10), and some of the small G proteins (see below), have been implicated to be directly involved in modulating integrin-mediated cell adhesion.

H-Ras and c-Myc proteins play crucial roles in regulating proliferation and transformation in multiple cell types (11, 12). It has been shown that H-Ras and c-Myc cooperate to induce cellular transformation in vitro and tumorigenesis in vivo (13–15). However, the roles of H-Ras and c-Myc, and in particular their cooperative roles in regulating cell adhesion, remain largely unknown. Involvement of small GTP-binding proteins in the regulation of cell adhesion has been suggested from an early observation that injection of GTP analogues into Xenopus XTC fibroblasts inhibits ruffling and increases cell spreading (16). Recent studies further demonstrate participation of H-Ras and R-Ras in regulating the activity of integrins via inside-out signaling. It has been reported that ectopic expression of an active form of H-Ras in Chinese hamster ovary (CHO) cells, stably expressing a chimeric integrin, suppressed the function of the chimeric integrin (17). In contrast, expression of an active form of R-Ras, which is related to H-Ras, has been found to enhance cell adhesion to the extracellular matrix via activation of several integrins (18). In contrast, previous studies demonstrate that Rho plays essential roles in regulating cytoskeletal organization and adhesive activity (19–22). Furthermore, it has been shown that the effects of Ras on cytoskeletal organization, cell adhesiveness, and proliferation are mediated by Rho signaling pathway (23–25). However, little is known about the function of c-Myc in modulating cell adhesion, except for an...
observation that c-Myc can down-regulate the LFA-1 adhesion receptor (26).

In this study, we investigated the effects of protooncoproteins H-Ras and c-Myc on hematopoietic cellular behavior using hematopoietic progenitor BAF-B03 cells and found that constitutive expression of an active form of H-Ras (H-RasV12), but not c-Myc alone, enhances cell adhesion to Fn by activating VLA-4 without alteration of its surface expression. Interestingly, coexpression of H-RasV12 and c-Myc induces a homotypic cell adhesion, which is mediated, at least partly, by the interaction between activated VLA-4 integrin and inducibly expressed VCAM-1. Hence, it becomes evident that H-Ras can regulate cell adhesion molecules through c-Myc-dependent and -independent mechanisms.

Materials and Methods

Abs and reagents

Monoclonal Abs used in this study are as follows: HMα1 (anti-α1; Ref. 27), HMα2 (anti-α2; Ref. 27), PS2/4 (anti-α4; Ref. 28), HMα5-1 (anti-α5; Ref. 29), KBA (anti-α7; Ref. 30), Mac-1 (anti-β2; Ref. 31), RMV-7 (anti-β7; Ref. 32), HMβ1-1 (anti-β1; Ref. 33). 9EG7 (anti-β3; Ref. 34), M18/2 (anti-β2; Ref. 31), HMβ3-1 (anti-β3; Ref. 35), M203 (anti-β5; Ref. 36), KAT-1 (anti-ICAM-1; Ref. 37), and MK-2 (anti-VCAM-1; Ref. 38). Anti-Thy-1 mAb was provided by Dr. E. Shevach (National Institutes of Health, Bethesda, MD). FITC-conjugated goat anti-rat and goat anti-hamster IgG were purchased from Cappel Laboratories (Malvern, PA). Phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204) Ab and phospho-specific p38 MAP kinase (Thr180/Tyr182) Ab were obtained from New England Biolabs (Beverly, MA). PD98059, a specific inhibitor of MAP/extracellular signal-related kinase (ERK) kinase (MEK) 1, was obtained from New England Biolabs. SB202190, a potent inhibitor of p38 MAP kinase, and SB202474, a negative control compound for p38 MAP kinase inhibition studies, were obtained from Calbiochem (San Diego, CA).

Cells and cell culture

BAF-B03, a subclone of the Ba/F3 cell line, is a bone marrow-derived murine IL-3-dependent pro-B cell line (39). BRV12 cells were established by transfecting an active form of human Ras expression plasmid, pEF-BOS-Ha-RasV12 (40) into BAF-B03 cells; BM cells were obtained by transfecting a human c-Myc expression plasmid (pN-LTR-myc; Ref. 41) into BAF-B03 cells; BMRV12 cells were established by transfecting pEF-BOS-Ha-RasV12 into BM cells. For all cell lines, at least three independent clones were established, and the results from a representative clone are shown. BMRV12 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, and other cells were cultured in the same RPMI 1640 medium containing 10% (v/v) WEHI-3B culture supernatant as a source of IL-3.

DNA transfection

Plasmid DNAs were transfected into cells by an electroporation procedure as described previously (42). Selection was initiated 24 h after DNA transfection using 2 μg/ml G418 for BRV12 and BMRV12 and 1 μg/ml hygromycin for BMRV12 cells. Drug-resistant clones were either pooled or subsequently cloned by limiting dilution as described previously (43).

Western blot analysis

Cells (5 × 10⁶) were harvested and solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 10 μg/mg PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) by sonication. Postnuclear supernatants were prepared by centrifugation at 10,000 × g for 10 min. Protein was quantified using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). For Western blot analysis, samples containing equal amounts of protein were subjected to SDS-PAGE (10% polyacrylamide gel). Separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). After blocking with TBST-milk (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween 20, 5% nonfat dry milk), membranes were incubated with anti-hemagglutinin (HA) mAb, 12CA25 (Boehringer Mannheim, Mannheim, Germany) 1:1000 dilution in TBST-0.5% milk overnight at 4°C. Then, membranes were washed with TBST and incubated with HRP-conjugated secondary Abs (1:3000 dilution in TBST-0.5% milk) for 1 h at room temperature. After three washes in TBST, proteins were detected using the enhanced chemiluminescence kit according to the manufacturer’s instructions (Amersham, Buckinghamshire, U.K.).

Flow cytometry

Cell-surface expression of adhesion molecules was analyzed by immunofluorescence using mAbs against the respective molecules as described previously (44). For each sample, a total of 1 × 10⁶ cells were treated with the respective mAbs for 30 min at 4°C. After washing, cells were stained with FITC-conjugated goat anti-rat or anti-hamster Abs. The stained cells were analyzed using a Coulter Epic XL-MCL flow cytometer (Coulter, Miami, FL).

Ab-blocking assay

Aggregated BMRV12 cells were mechanically separated into a suspension of single cells. For each sample, 5 × 10⁵ cells were replated in a 24-well plate (1 ml/well) along with various mAbs at a saturating concentration of 10 μg/ml, which was shown in previous studies to produce a maximum inhibition of the relevant adhesive interaction (45). The effect of mAb on cell aggregation was evaluated by observation of photomicrographs after 1–4 h incubation.

Cell adhesion assay

Adhesion assay of BAF-B03, BM, BRV12, and BMRV12 cells to Fn was performed essentially as previously described (46). Fn (5 μg/well; Seikagaku, Tokyo, Japan) or control 3% human serum albumin (Green-Cross, Osaka, Japan) was applied to a 48-well plate in PBS at 4°C overnight. Wells were subsequently blocked with Ca2+/Mg2+-free PBS/3% human serum albumin buffer at 2 ml at 37°C. After the plates were washed three times with PBS, 2 × 10⁵ BAF-B03 or transfectedants labeled with 51Cr (DuPont NEN, Wilmington, DE) were added to each well with or without blocking mAb (10 μg/ml) in the presence or absence of PMA (10 ng/ml; Sigma, St. Louis, MO). To examine the effects of MEK1 inhibitor or p38 inhibitor, cells were pretreated with PD98059 (50 μM), SB202190 (50 μM), or SB202474 (50 μM) for 1 h. After settling for 30 min at 4°C, the plates were rapidly warmed to 37°C for 30 min, then gently washed twice with RPMI 1640 at room temperature to completely remove nonadherent cells. The adherent cells contained in each well were lysed with 250 ml of 1% Triton X-100, and the 51Cr radioactivity was measured using a gamma-counter. Data were expressed as mean percentage of the binding of indicated cells from a representative experiment.

Cell growth assay

For cell growth assay, factor-independent BMRV12 cells were cultured at a density of 5 × 10³ cells/ml in RPMI 1640 supplemented with 10% FCS, and other cells were cultured in the same RPMI 1640 medium containing 10% WEHI-3B supernatant as a source of IL-3. The culture medium was

![FIGURE 1. Proliferation and viability profiles of BAF-B03-derived cells. Cells were plated at 5 × 10⁶ cells/ml in the presence or absence of IL-3. The concentration of viable cells was counted at various time points after plating and are represented on a logarithmic scale. Expression of exogenous H-RasV12 was detected by Western blot analysis using anti-HA mAb (inset). Abbreviations: BM, BAF-B03 cells expressing human c-Myc alone; BRV12, BAF-B03 cells expressing human H-RasV12 alone; BMRV12, BAF-B03 cells expressing both human H-RasV12 and c-Myc.](Image 315x574 to 528x734)
changed every other day. For the cell viability assay, BAF-B03, BM, and BR V12 cells were washed with PBS to remove cytokines and then cultured at a density of $5 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% FCS. Viable cell numbers were determined with a trypan blue exclusion assay.

**Actin polymerization**

The presence of F-actin was detected as described previously (47). In brief, cells ($10^6$/ml) were fixed on slides and then permeabilized. F-actin was detected by being stained with rhodamine-phalloidin (1 U/slide; Molecular Probes, Eugene, OR) and was analyzed by a confocal laser scan microscope system (LSM410UV, Carl Zeiss, Oberkochen, Germany).

**Northern blot analysis**

Total RNAs from cells were prepared by using Isogen (Wako, Osaka, Japan). For RNA blot analysis, 10 μg of total RNA was electrophoresed on 1% agarose formaldehyde gels and transferred onto nylon membranes. The probe DNA (~1.4 kb) was prepared from pCR2.1-TOPO-mouse VCAM-1 (nt 733-2200 of the open reading frame) by digestion with EcoRI and labeled with $[\alpha-^32P]dCTP$ (3000 Ci/mm; Amersham) using the Multi-prime labeling kit (Amersham) and hybridized as described previously (44). Specific activity was $-1 \times 10^6$ cpm/ng for the probe DNA.

**Results**

**Cell adhesion of BAF-B03 cells is induced by ectopic expression of H-Ras V12 singly or in combination with c-Myc**

As an attempt to investigate the effect of protooncoproteins H-Ras and c-Myc on hemopoietic cellular behavior, an active form of the human H-ras (H-rasV12) and c-Myc were stably expressed singly or in combination in an IL-3-dependent mouse pro-B cell line, BAF-B03, which normally grows as a suspension of single cells. We noticed that BAF-B03 cells became slightly adhesive to culture plates after transfection with an expression plasmid encoding an active form of human H-RasV12 (termed BR v12) (data not shown), yet the BR v12 cells still required IL-3 for their proliferation. Interestingly, BAF-B03 cells expressing both active human H-Ras V12 and human c-Myc (termed BMR V12 cells) were able to proliferate in a cytokine-independent fashion (Fig. 1) as well as to form cell aggregates (Fig. 2A) despite the fact that cells expressing human c-Myc alone (BM cells) failed to display analogous behavior (Figs. 1 and 2A). Furthermore, we examined the actin polymerization in these transfectants as well as parental BAF-B03 cells. Remarkable actin polymerization was observed in BR V12 and

![FIGURE 2. A, Morphological properties of BAF-B03-derived cells. BAF-B03, BM, and BR V12 cells were cultured in the presence of IL-3 while BMR V12 cells were cultured in the absence of IL-3. The addition of IL-3 did not alter the cell adhesion property of BMR V12 cells. B, Induction of actin polymerization in BR V12 and BMR V12 cells. BAF-B03 (+IL-3), BR V12 (+IL-3), and BMR V12 (-IL-3) cells were stained with rhodamine-phalloidin to detect F-actin. Remarkable actin polymerization was observed in BR V12 and BMR V12 cells, while weak staining was detected in BAF-B03 cells. Quantitational analysis revealed that intensities of rhodamine-phalloidin staining of BR V12 and BMR V12 cells were about 2.7- and 2.5-fold higher than those of BAF-B03 cells, respectively. The magnification of each panel is ×3427. The size of BAF-B03 and BR V12 cells is somewhat smaller and larger than that of BMR V12 cells, respectively.](http://www.jimmunol.org/)

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BMR<sup>V12</sup> cells, but not in BAF-B03 (Fig. 2B) and BM cells (data not shown). These observations suggested that H-Ras<sup>V12</sup>, by itself or in cooperation with c-Myc, may play an important role in the regulation of certain cell adhesion molecule(s) and cytoskeletal molecule(s).

Expression of VCAM-1 is induced by a cooperative function of H-Ras<sup>V12</sup> and c-Myc

To determine which molecules might be responsible for the observed adhesive properties of BR<sup>V12</sup> and BMR<sup>V12</sup> cells, candidate molecules were sought using a panel of Abs and flow cytometric analysis (see Materials and Methods). The expression of α<sub>1</sub>, α<sub>2</sub>, α<sub>4</sub>, α<sub>5</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2</sub>, and β<sub>7</sub> chains was not detectable on either BAF-B03, BM, or BR<sup>V12</sup> and BMR<sup>V12</sup> cells (Fig. 3A). We found that the integrin α<sub>5</sub> and β<sub>1</sub> chains as well as ICAM-1 (Fig. 3A) are comparably expressed on BAF-B03, BR<sup>V12</sup>, BM, and BMR<sup>V12</sup> cells. The expression of the integrin α<sub>1</sub> chain was also detectable on these cells, although its expression level on BMR<sup>V12</sup> cells was somewhat lower than the others (Fig. 3B, left). Noticeably, VCAM-1 is not expressed on either the BAF-B03, BR<sup>V12</sup>, or BM cells, but is expressed substantially on the surface of BMR<sup>V12</sup> cells (Fig. 3B, right). Furthermore, consistent with the result obtained by flow cytometric analysis, Northern blot analysis revealed that a high level of VCAM-1 transcripts was detected in BMR<sup>V12</sup> cells, although the expression of VCAM-1 transcripts was hardly detectable in the BAF-B03, BR<sup>V12</sup>, or BM cells (Fig. 3C). This results indicated that expression of H-Ras<sup>V12</sup> or c-Myc alone is insufficient for the induction of VCAM-1 and that a cooperative effect of H-Ras<sup>V12</sup> and c-Myc is required for the induction of VCAM-1 expression.

Both VLA-4 (α<sub>β1</sub>) and VCAM-1 are involved in homotypic cell adhesion induced by coexpression of H-Ras<sup>V12</sup> and c-Myc

To identify the adhesion molecules mediating the homotypic aggregation of BMR<sup>V12</sup> cells, we examined the effects of several function-blocking Abs on the homotypic aggregation of the cells. It was found that homotypic aggregation of BMR<sup>V12</sup> cells was almost completely inhibited by either anti-α<sub>4</sub> (PS/2) or anti-VCAM-1 (M/K2) mAbs, whereas anti-α<sub>4</sub> (KBA), anti-ICAM-1(KAT-1), anti-α<sub>6</sub>, (RMV-7), or a control nonblocking anti-β<sub>1</sub> (M18) mAbs failed to inhibit homotypic aggregation of BMR<sup>V12</sup> cells (Fig. 4A). Thus, the results suggested that α<sub>4</sub> integrin may be activated on these cells, and that both α<sub>4</sub> integrin and VCAM-1 are primarily involved in the homotypic aggregation of BMR<sup>V12</sup> cells. Although the integrin α<sub>1</sub> chain is capable of associating with either β<sub>1</sub> (α<sub>β1</sub>, VLA-4) or β<sub>7</sub> (48) to mediate adhesion to VCAM-1 or Fn, the β<sub>1</sub> but not the β<sub>7</sub> subunit is expressed on BAF-B03 cells and their derived transfectants (Fig. 2A), suggesting that α<sub>4</sub> associates with β<sub>1</sub> to form the heterodimer (VLA-4) on these cells. Noticeably, an anti-β<sub>1</sub> mAb, HMβ1–1, which has been shown to block the binding of α<sub>4</sub>β<sub>1</sub> to Fn (33), failed to inhibit homotypic aggregation of BMR<sup>V12</sup> cells under our experimental conditions (up to 50 μg/ml, data not shown), and it is likely that HMβ1–1 fails to recognize the epitope on the β<sub>1</sub> that is required for the interaction with VCAM-1. In fact, it was shown that the sites within the β<sub>1</sub> integrin involved in the ligation of VLA-4/VCAM-1 and VLA-4/Fn are different (3). Importantly, anti-mouse β<sub>1</sub> mAb, 9EG7 (34), which recognizes the ligand-binding or activated epitope of the β<sub>1</sub> integrin, considerably discriminates BR<sup>V12</sup> and BMR<sup>V12</sup> cells from BAF-B03 and BM cells. As shown in Fig. 4B, the ligand-binding (or activated) epitope of β<sub>1</sub> is induced on BR<sup>V12</sup> and BMR<sup>V12</sup> cells at higher levels compared with BAF-B03 or BM cells as assessed by flow cytometric analyses.
suggesting that expression of H-RasV12 alone is sufficient to activate β1 integrin. Collectively, our results indicate that VLA-4 and VCAM-1 are primarily responsible for the homotypic aggregation of BMRV12 cells.

Increased cell adhesion to Fn by H-RasV12 is independent of c-Myc

Because the activated β1 is detectable on BRV12 and BMRV12 cells among cells examined, we assessed the adhesive abilities of integrin β1 on BRV12 and BMRV12 cells to Fn coated on plates. It was found that BMRV12 and BRV12 cells can bind to Fn efficiently. Binding of BRV12 and BMRV12 cells to Fn was augmented about 6- and 4-fold over the control, respectively (Fig. 5A). Furthermore, the addition of phorbol ester, a potent integrin trigger, resulted in apparently enhanced adhesion of BAF-B03 cells to Fn, whereas the adhesion of BRV12 and BMRV12 cells to Fn was only moderately and weakly augmented, respectively (Fig. 5A), indicating that H-RasV12 can play a pivotal role in the activation of integrin. The fact that attachment of BMRV12 cells to Fn was weaker than that of BRV12 cells might reflect the down-regulation of VLA-4 integrin on BMRV12 cells by H-Ras and c-Myc (see Discussion). In contrast, no enhanced attachment of BAF-B03 (Fig. 5A), BM, BRV12N (BAF-B03 cells expressing a dominant negative form of H-Ras, H-RasV12N), and BAF-B03 cells transfected with an empty vector to Fn was observed (data not shown). Adhesion of BRV12 and BMRV12 cells to Fn was selectively inhibited by the anti-α4...
or treated with PD98059 (50 μM) as described in Materials and Methods. Mean percentages of binding are shown. Essentially identical results were obtained in three independent experiments.

A. Effect of anti-α4 blocking mAb (PS/2) on the adhesion of BAF-B03 cells and of their derived transfectants to Fn. The indicated cells were untreated or treated with anti-α4 integrin mAb as described in Materials and Methods. Mean percentages of binding are shown. Essentially identical results were obtained in three independent experiments.

B. Effect of MEK1 inhibitor (PD98059) or p38 inhibitor (SB202190) on the adhesion of BAF-B03 and BR v12 cells to Fn. The indicated cells were untreated or treated with PD98059 (50 μM), SB202190 (50 μM), or with SB202474 (50 μM) as described in Materials and Methods. Mean percentages of binding are shown. Essentially identical results were obtained in independent experiments. Immunoblot analysis with phospho-ERK and phospho-p38 MAP kinase Abs revealed that PD98059 and SB202190 almost completely inhibit ERK and p38 activities, respectively, under our experimental conditions (data not shown).

mAb (PS/2), but not by an irrelevant anti-Thy-1 mAb (Fig. 5A). However, the inhibition by the anti-α4 mAb was incomplete (~50%), suggesting that other integrins such as α5 may also be activated on these cells. Collectively, these results indicate that VLA-4 expressed on the surface of both BR v12 and BMR v12 cells are dominantly activated by H-Ras v12+, and thus VLA-4 activation is a c-Myc-independent process.

It was found that both ERK1 and ERK2 are activated constitutively in BR v12 cells (data not shown). Thus, we examined whether or not MEK/ERK (or p38 MAP kinase) is involved in H-Ras v12+-mediated activation of VLA-4 in BR v12 cells by using the MEK inhibitor (PD98059) or p38 inhibitor (SB202190). It was found that PD98059, SB202190, and SB202474 (negative control compound) have marginal effects on the binding of BAF-B03 and BR v12 cells to Fn (Fig. 5B). Furthermore, the addition of PD98059 or SB202190 failed to inhibit the homotypic aggregation of BMR v12 cells (data not shown). These results suggest that MEK/ERK as well as p38 MAP kinase are not involved in H-Ras v12+-mediated activation of VLA-4.

Discussion

Activation of VLA-4 by H-Ras is a particularly surprising observation, because an inhibitory effect of H-Ras on certain integrins has been recently reported (17). However, it was unclear whether some types of integrins, such as VLA-4, were actually activated by H-Ras in their study, because a constitutively active chimeric integrin system was employed to monitor the suppressive effect rather than to detect activated function. The suppression of integrin function by H-Ras has been shown to be mediated through the Ras/Raf/MAPK (ERK) pathway (17). Interestingly, although both ERK1 and ERK2 are activated constitutively in BR v12 cells (data not shown), it was suggested that ERKs are not involved in activation of VLA-4 in the cells (Fig. 5B). One plausible explanation for such a discrepancy is that H-Ras may possess distinct functions in regulating different types of integrins, although cell type-specific functions of H-Ras have also to be considered. In contrast, Zhang et al. have reported that another Ras family member, R-Ras, is able to activate integrins (18). R-Ras is a GTP-binding protein highly homologous to H-Ras protein, but has an additional 26 aa at the amino terminus (49). R-Ras and H-Ras have similar effector binding domains and bind to many identical effectors, including Raf and Ral-GDS (50). It is of interest to examine whether R-Ras-mediated activation of integrins also involve the activation of Raf/MAPK pathway.

While the activation of VLA-4 on BAF-B03 cells relies solely on H-Ras v12+, induction of VCAM-1 by H-Ras v12+ is a c-Myc-dependent process. In this respect, it is of importance to note that ectopic expression of H-Ras v12+ itself did not affect the expression of c-Myc and vice versa (data not shown). At present, the mechanism of c-Myc-dependent expression of VCAM-1 remains unclear. It is unlikely that c-Myc can directly regulate VCAM-1 expression through its activity as a transcription factor, because c-Myc binding sequences have not been reported within the promoter region of VCAM-1. One possible mechanism of the VCAM-1 induction is that H-Ras and c-Myc act cooperatively to induce some cytokines, such as TNF-α, IL-1α, and IL-4, which are known to be able to induce VCAM-1 expression (51–53). To test this, we used the supernatants of growing BMR v12 cells to culture either parental BAF-B03 or BM and BR v12+ cells and found that expression of VCAM-1 on these cells was not induced under our experimental conditions and that BR v12+ cells did not form aggregates (data not shown), indicating that induction of VCAM-1 is not due to indirect cytokine stimulation. Further study will be required to elucidate the molecular basis of VCAM-1 induction by H-Ras v12+ and c-Myc.

Although the c-Myc-dependent mechanism is primarily responsible for the induction of VCAM-1, the function of c-Myc appears not to be so simple. c-Myc may also participate in the down-regulation of VLA-4 expression on BMR v12+ cells in collaboration with H-Ras v12+, 1) expression of the α4 integrin on BMR v12+ cells is partly (~30%) down-regulated compared with that on BAF-B03, BM, or BR v12+ cells (Fig. 3B); 2) although expression of total β1 on BR v12+ and BMR v12+ cells is comparable, the amount of activated β1 on BMR v12+ cells is partly (~30%) down-regulated compared with that on BR v12+ cells (Fig. 4B); 3) attachment of BMR v12+ cells to Fn is weaker than that of BR v12+ cells (Fig. 5A). Collectively, these results suggest that VLA-4 expression as well as VLA-4 activity on BMR v12+ cells may be down-regulated. The evidence of a possible negative regulatory effect of c-Myc on VLA-4 is reminiscent of the previous report, showing that c-Myc could down-regulate LFA-1 (26). In addition, down-regulation of integrins by N-myC has been demonstrated (54, 55). It has been reported that integrin function (or expression) is often diminished upon oncogenic transformation (56, 57). Hence, down-regulation of VLA-4 integrin observed on BMR v12+ cells may reflect the cellular transformation of BMR v12+ cells. In fact, we found that BMR v12+ cells proliferate in a cytokine-independent manner (Fig. 1) and form foci in soft agar in the absence of IL-3 (data not shown). Thus, our results may also

FIGURE 5. A. Effect of anti-α4 blocking mAb (PS/2) on the adhesion of BAF-B03 cells and of their derived transfectants to Fn. The indicated cells were untreated or treated with anti-α4 integrin mAb as described in Materials and Methods. Mean percentages of binding are shown. Essentially identical results were obtained in three independent experiments. B. Effect of MEK1 inhibitor (PD98059) or p38 inhibitor (SB202190) on the adhesion of BAF-B03 and BR v12 cells to Fn. The indicated cells were untreated or treated with PD98059 (50 μM), SB202190 (50 μM), or with SB202474 (50 μM) as described in Materials and Methods. Mean percentages of binding are shown.
provide a possible mechanism to explain such down-regulation of integrins upon cellular transformation.

Simultaneous activation of VLA-4 and expression of VCAM-1 on the same cell is an interesting phenomenon. It was originally supposed that these two processes are regulated by distinct mechanisms in different types of cells, because the cellular and tissue distributions of VLA-4 and VCAM-1 are quite different and VLA-4/VCAM-1 generally mediates heterotypic cell-to-cell interactions, such as those between leukocytes and endothelial cells. An interesting example of a VLA-4/VCAM-1 interaction occurring on the same types of cells is the observation of Rosen et al. (58) that VLA-4 and VCAM-1 are expressed concomitantly on myoblasts. The VLA-4/VCAM-1 interaction has been suggested to be crucial for myogenesis. However, in the above case, activity of VLA-4 is regulated at the expression level rather than modulation of its ligand-binding activity (affinity) by inside-out signaling. In addition, it remains unclear which intracellular molecules are responsible for this regulation.

Importantly, we have recently observed that BAF-B03 cells expressing both cyclin C and c-Myc (term BMC cells) exhibit essentially identical cellular behaviors with BMRv12 cells (59). It can be assumed that cyclin C may be one of candidate downstream targets of H-Ras, because cyclin C, like H-Ras, is able to cooperate with c-Myc to induce cytokine-independent growth and homotypic adhesion of BAF-B03 cells. However, unlike H-Ras, ectopic expression of cyclin C alone in BAF-B03 cells fails to activate VLA-4. It is perhaps due to that cyclin C may be just one of multiple H-Ras targets required for VLA-4 activation, or it simply reflects the difference in the functional strength between constitutively active H-Ras (H-Ras12) and the wild-type cyclin C. By contrast, it seems unlikely that the function of cyclin C is mediated by the activation of endogenous H-Ras, because overexpression of a dominant negative form of H-Ras (H-RasS17N) in BMC cells, where cyclin C as well as c-Myc are expressed ectopically at high levels, fails to affect their cell adhesion properties (data not shown). It is also possible that cyclin C and H-Ras may use distinct mechanisms to mediate activation of VLA-4 and expression of VCAM-1 in cooperation with c-Myc.

The physiological and pathological significance of the regulation of the functional properties of VLA-4 and VCAM-1 on hematopoietic cells remains to be determined. Activation of VLA-4/VCAM-1 pair on the hematopoietic progenitors may be of importance for the regulation of hemopoiesis. Adhesive interactions of VLA-4 with VCAM-1 on stromal cells or with extracellular matrix retain hematopoietic progenitor cells in close vicinity to components of the bone marrow microenvironment that are required for the regulation of physiological hemopoiesis. The importance of VLA-4 in hemopoiesis has been proved by the fact that the addition of anti-cα4 mAb to long-term bone marrow cultures abrogated lymphopoiesis and retarded myelopoiesis (28). VLA-4-specific Abs have also been shown to abrogate stroma-dependent erythropoiesis (29). VCAM-1 may also contribute to promote lympho- and myelopoiesis (5, 60, 61). In addition, VLA-4/VCAM-1-mediated cell adhesion has been assumed to play an important role(s) in the migration of leukocytes (62, 63) and circulating malignant cells (64, 65), which is a critical step in the process of inflammation and metastasis. The elucidation of the c-Myc-dependent and -independent functions of H-Ras in governing cell adhesion warrants further study in the context of gaining insights into the pathophysiologic mechanisms regulating multiple biological processes, such as hemopoiesis, differentiation, inflammation, and metastases, in which VLA-4 integrin and VCAM-1 play essential roles.

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References


