The cell adhesion molecule L1 has been implicated in a variety of motile processes, including neurite extension, cerebellar cell migration, extravasation, and metastasis. Homophilic or heterophilic L1 binding and concomitant signaling have been shown to promote cell motility in the short term. In this report, L1 is also shown to induce and maintain a motile and invasive phenotype by promoting gene transcription. In the presence of serum or platelet-derived growth factor, L1 promotes heightened and sustained activation of the extracellular signal-regulated kinase pathway. Activation of this pathway then induces the expression of motility- and invasion-associated gene products, including the \(\beta_3\)-integrin subunit, small GTPases, and the cysteine protease cathepsin-L and -B. Induction of integrin \(\alpha_\beta_3\) and rac-1 is shown to contribute directly to L1-dependent haptotaxis, whereas induction of cathepsins-L and -B promotes matrix invasion. This study provides a novel translational mechanism to account for the association between L1 expression and motile processes involved in metastasis and development.

The L1 cell adhesion molecule is a phylogenetically conserved neural recognition molecule that belongs to the immunoglobulin superfamily. Structurally, L1 is a transmembrane (type 1) glycoprotein with a complex ectodomain consisting of multiple immunoglobulin and fibronectin-like repeats (1, 2). L1 was first described as a neural cell adhesion molecule based upon a restricted distribution that included post-mitotic neurons (3). However, both murine and human L1 homologues have now also been described for other cell types of diverse origin, including endothelial cells, epithelial cells, reticular fibroblasts, and cells of lymphoid and myelomonocytic origin (4–10).

Although L1 serves to promote cell-cell interactions, the functional outcome of such interactions is rarely static cell-cell adhesion. In the central nervous system, for example, L1 has been shown to initiate a variety of dynamic motile processes that include cerebellar cell migration and neurite extension (3, 11–13). Function-blocking antibodies to L1 have been shown to abrogate the migration of granule precursors in cerebellar explant cultures (3, 14), and the migration and positioning of dopaminergic neuronal precursors is disrupted in L1-deficient mice (13). Additional studies have linked L1 to motile processes involved in tumor cell extravasation and glioma dissemination in the brain (15, 16). Several recent reports have also now linked L1 expression to melanoma and prostate metastasis (17–20). For example, Thies et al. (18) demonstrated that L1 expression in primary melanoma is a highly significant indicator for subsequent metastasis and reduced patient survival.

Cell-cell interaction and homophilic L1-ligation has been shown to be an important stimulus for cell motility (11). However, L1 is also now known to have a significant impact upon cell-matrix interactions required for haptotaxis and matrix remodeling (9, 21, 22). Two recent reports (21, 22) have shown that ectopic L1 expression strongly potentiates \(\alpha_\beta_3\)-mediated haptotaxis. In both of these studies, initiation of haptotaxis is proposed to involve direct L1-integrin interaction via an Arg-Gly-Asp integrin-recognition motif in the L1 ectodomain. Such an association may depend upon a transient cis-association at the cell surface (22) or involve L1-shedding and autocrinal binding via \(\alpha_\beta_3\) (21). In both studies, it is suggested that direct L1-integrin ligation potentiates haptotaxis via non-translational mechanisms that involve the induction of integrin-mediated signaling events (21, 22).

In this report, we present evidence for a translational mechanism that can further account for the association between L1 expression and cell motility and invasion. Ectopic L1 expression in two different cell lines is shown to induce sustained activation of the extracellular signal-regulated kinase (ERK) pathway and the concomitant induction of ERK-regulated gene products intimately associated with cell motility and invasion. Several of these gene products are shown to contribute directly to L1-mediated migration and matrix invasion. Such a mechanism may allow L1 to induce and maintain a motile cellular phenotype that facilitates both normal developmental repositioning and metastasis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-mouse integrin antibodies to \(\beta_3\) (Ha25), \(\alpha_5\) (Ha31/8), \(\alpha_5\) (HM22), \(\alpha_5\) (5H10–27), \(\alpha_5\) (GoH3), \(\beta_3\) (2C9.G2), and \(\alpha_6\) (H9.2b8) were purchased from BD Biosciences (San Diego, CA). The inhibitory reagent PDGF, cathepsin-L, and cathepsin-B are available from R&D Systems (Minneapolis, MN). mAb, monoclonal antibody; pAb, polyclonal antibody; PAGS, fluorescein-activated cell sorting; PDGF, platelet-derived growth factor; 4HT, 4-hydroxytamoxifen; RBM, reconstituted basement membrane.

4 The abbreviations used are: ERK, extracellular signal-regulated kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGS, fluorescein-activated cell sorting; PDGF, platelet-derived growth factor; 4HT, 4-hydroxytamoxifen; RBM, reconstituted basement membrane.
purchased from BD Biosciences. A goat polyclonal antibody (pAb) to α2β1 (FNIII) was obtained from Chemicon Inc. (Temecula, CA). Anti-phospho-ERK monoclonal antibody (mAb) (E10) and anti-ERK-2 pAb (C-14) were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-L1 mAb (U1212) was from Neomarkers (Fremont, CA) and an anti-L1 pAb (L1-ECID) was kindly provided by Dr. William Stallcup (Burnham Institute, La Jolla, CA). Anti-thrombospondin-2 mAb Cl.4 and anti-Rac1 mAb (Cl.102) and anti-S100A4 pAb were obtained from BD Transduction Labs and DAKO (Glostrup, Denmark), respectively. The mitogen-activated protein/ERK kinase inhibitor U0126 was purchased from Promega (Madison, WI). Purified vitronectin was obtained from Chemicon; laminin from BD Biosciences; purified type I collagen from Upstate Biotechnology (Lake Placid, NY); and human type IV collagen from Sigma.

Cell Models and Transfections—NIH-3T3 cells used for stable and transient transfections were obtained from the American Type Culture Collection (Rockville, MD). 3T3 cells stably expressing L1 were provided by Dr. V. Lemmon (University of Miami) and were generated by using LipofectAMINE and a pcDNA3 vector containing full-length L1 cDNA (23). Mock-transfected 3T3 cells were generated using LipofectAMINE and an empty pcDNA3.1/neomac2 vector. Drug-resistant L1- and mock-transfected 3T3 cell populations were cultured in Dulbecco’s modified Eagle’s medium containing G418 (800 μg/ml) and 10% fetal calf serum and were routinely passaged to avoid overgrowth. The cloned melanoma cell line K1735-C11 was provided by Patricia Ray (Wayne State University). To produce stable transfecients, the cells were transfected using LipofectAMINE Plus reagent (Invitrogen) and FVU-linearized pcDNA3.1(zeo) vector encoding full-length human L1. Transfected cells were selected and maintained in Dulbecco’s modified Eagle’s medium containing Zeocin (800 μg/ml) and 10% fetal calf serum. L1-expressing cells were sorted by repeated fluorescence-activated-cell sorter (FACS) analysis with an anti-L1 pAb. Transfected, non-expressing mock control cells were maintained under identical culture conditions and were used after an equal number of passages.

For the transient transfection of L1, subconfluent 3T3 cells were incubated with LipofectAMINE Plus reagent and a pcDNA3.1 expression vector (Invitrogen) incorporating the entire 3.4-kb 3’ end of the full-length L1 complementary DNA sequence. Empty vector was used for mock transfection. After 5 h, the cells were washed twice and replenished with fresh serum-containing media. Cells were lysed for Western blot analysis after an additional 72 h. L1-transfected 3T3 cells were also transiently transfected with a dominant-negative hemagglutinin-tagged Rac1 expression construct containing an asparagine mutation of Thr17 (Rac-N17) (24). Control cells received empty vector, and all cells were cotransfected with a β-galactosidase reporter vector (pEF4-lacZ). Cells were transfected with LipofectAMINE Plus reagent as described above. After 48 h, cells were harvested and assessed for migration; 5-bromo-4-chloro-3-indolyβ-D-galactopyranoside (X-gal) staining within adherent cells was enzymatically cleaved by β-galactosidase, and the X-gal-positive cells were quantified by counting the number of cells per 20× field using an inverted stereomicroscope. Eight fields per insert were scored, and treatments were performed in duplicate. For inhibition studies, anti-integrin antibody (35) (BD BioCoat growth factor reduced Matrigel invasion chambers as recommended by the manufacturer (BD Biosciences). Cells were added at 5 × 105 cells per upper chamber in fibroblast basal media supplemented with 5% bovine serum albumin and 0.4 mM MnCl2. These cells were grown to 90% confluence 18 h prior to use and were harvested from cultures at 70–80% confluence using versene. Invasion of cells from upper to lower chambers was determined after 22–42 h and quantified by counting cells on the undersides of the insert membranes as described for migration assays. PDGF (50 ng/ml) was added to lower chambers as a chemorepellent. The contribution of cpehains to invasion was assessed using peptides CA-064 Me (selective inhibitor of cathepsin-B) and E-64-d (inhibitor of cpehains-L and -B) (Peptide International Inc, Louisville, KY). These peptides were added to upper and lower chambers at concentrations ranging from 1.5 to 25 μM. To optimize cpehain activity, the invasion fibroblast basal medium was adjusted to pH 6.8.

Flow Cytometry—Surface integrin expression was assessed by FACS analysis. Sub-confluent cultures were harvested using versene and stained with the following anti-integrin antibodies: anti-β1 (CD29, Ha2/5), anti-α5 (CD49a, Ha3/18), anti-α6 (CD49b, HMa2), anti-αv (CD49f, GoH3), anti-β1 (CD61, C29.G2), and anti-αv (CD61, Hb.28). Anti-α5, α6, and β1 antibodies were all conjugated directly to phycoerythrin. Intracellular cathepsin-L and -B activity was measured using Magic Red TM cathepsin detection kits for cathepsin-L and -B (Immunochemistry Technologies, LLC). Because of the high levels of intracellular cpehin-L and -B activity in 3T3 cells, the fluorogenic substrate for cpehain-B was diluted 1:300, which is below the manufacturer’s recommendations (Invitrogen). Ten percent of the cDNA synthesis Q10 kit (SuperArray, Inc., Bethesda, MD). Briefly, equal quantities of total RNA were hybridized to kit-specific primers, and gene expression was performed for 35 cycles with an annealing temperature of 55 °C using the HotStarTaq Mastermix Kit (Qiagen). Equal volumes of products were separated on a 1% agarose gel and documented by ethidium bromide staining. The following primer sets were used: forward, 5'-GTGCT-TTACCGCAGGAG-3' and reverse, 5'-TATGGTCTATTGGAGCAGCAGACG-3'. Actin primers were purchased from Stratagene. Amplification was performed for 35 cycles with an annealing temperature of 60 °C using the HotStarTag Mastermix Kit (Qiagen). Equal volumes of products were separated by agarose electrophoresis and documented with a Bio-Rad imaging system.

Invasion Assays—Matrigel invasion assays were performed using BD BioCoat growth factor reduced Matrigel invasion chambers as recommended by the manufacturer (BD Biosciences). Cells were added at 5 × 105 cells per upper chamber in fibroblast basal media supplemented with 0.5% bovine serum albumin and 0.4 mM MnCl2. These cells were grown to 90% confluence 18 h prior to use and were harvested from cultures at 70–80% confluence using versene. Invasion of cells from upper to lower chambers was determined after 22–42 h and quantified by counting cells on the undersides of the insert membranes as described for migration assays. PDGF (50 ng/ml) was added to lower
branes were blocked for 45 min prior to application of a horseradish peroxidase-labeled anti-biotin mAb (BN34, Sigma) in SuperArray wash buffer. The membranes were incubated for 30 min and washed vigorously with SuperArray wash buffer prior to rinsing with phosphate-buffered saline and chemiluminescent detection of bound probe with the horseradish peroxidase substrate PS-3 (Lumigen, Inc., Southfield, MI). Densitometric quantification of images was performed using both ScanAlyze and NIH Image software with similar results.

RESULTS

L1 Co-operates with Serum or Serum-growth Factors to Induce Sustained ERK Activation—Levels of constitutive ERK activation were assessed in 3T3 or K1735-C11 cells stably transfected with full-length human L1. L1 levels achieved in these cell lines are shown in Fig. 1A and are within the range of endogenous L1 expression displayed by a variety of cell lines (6, 25–27). Levels of ERK activation were determined in mock- or L1-transfected cells cultured in growth media and harvested at equivalent cell densities. Significantly higher levels of ERK-1/2 phosphorylation were observed as a result of stable ectopic L1 expression in both 3T3 and K1735-C11 cells (Fig. 1B). To ensure that the differences in ERK activation observed are due to L1 expression, rather than a drift in phenotype between mock- and L1-transfected populations, it was further confirmed that transient transfection of full-length L1 cDNA into wild-type 3T3 cells also induces higher levels of ERK activation (Fig. 1C).

The L1-mediated ERK activation observed was found to be critically dependent upon cell density. Thus, no evidence of L1-mediated ERK activation was observed when transfected 3T3 cells were seeded at a low cell density and were harvested as a dispersed or a sparse population (Fig. 1, D and E; 0.6 × 10^5/well). Differences in ERK activation were only evident at higher seeding densities (e.g. 2.5 × 10^5/well), which allowed for more contiguous monolayer formation and cell-cell contact (Fig. 1, D and E). After seeding at an optimal cell density, L1-dependent ERK activation was maintained for at least 24 h (Fig. 1F). After this time, the 3T3 cell cultures became excessively overgrown and ERK activation declined.

The ability of L1 to support sustained elevated ERK activation was also found to be critically dependent upon the presence of serum. Accordingly, no difference in ERK activation was found when the mock- or L1-transfected 3T3 cells were maintained in the absence of serum (Fig. 2A). One explanation for this finding is that sustained L1-dependent ERK activation requires co-operation with one or more serum growth factors. Because platelet-derived growth factor (PDGF) is a major mitogenic factor in serum, it was further determined whether L1 specifically co-operates with this growth factor to induce heightened levels of ERK activation. Importantly, levels of ERK activation resulting from stimulation with PDGF were significantly higher in 3T3 cells expressing L1 (Fig. 2, B and C). Together these data indicate that L1 can support sustained ERK activation; such a sustained response seems to require both cell-cell interaction and growth factor co-operation.

Ectopic L1 Expression and Sustained ERK Activation Induce Expression of Integrin α6β1—A prior report (28) has shown that
sustained activation of the ERK pathway induces the expression of select integrins, including $\alpha_6\beta_1$ and $\alpha_5\beta_1$. To determine whether L1 induces changes in integrin expression, we compared the integrin profiles of mock- or L1-transfected 3T3 cells. Cells were stained with antibodies to $\alpha_6\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_2$, $\alpha_5\beta_2$, and $\alpha_1$, and were then analyzed by FACS analysis.

A comparison of FACS histograms confirmed that stable expression of L1 in 3T3 cells is associated with a significant increase in the surface expression of $\alpha_6\beta_1$ (Fig. 3B). No change in the levels of the $\alpha_6\beta_2$-subunit, $\alpha_6\beta_3$, $\alpha_5\beta_1$, or $\alpha_5\beta_2$ was observed (Fig. 3C–F), and only a modest increase in the expression of $\alpha_6\beta_1$ and the $\alpha_6\beta_2$-subunit was found (Fig. 3G and H). Induction of $\alpha_6\beta_3$ expression was also confirmed in L1-transfected K1735 cells (Fig. 3I), and reverse transcription-PCR analysis, using primers specific for the murine $\beta_3$-subunit, established that the increased surface expression of $\alpha_6\beta_1$ in the 3T3 cells is associated with a significant increase in message for the $\beta_3$-subunit (Fig. 3J). Increased expression of the $\alpha_6\beta_3$-heterodimer (Fig. 3B), without a change in the overall levels of the $\alpha_6$-subunit (Fig. 3C), has been reported (28) and is believed to occur at the expense of other $\alpha_6$-heterodimers such as $\alpha_6\beta_2$ (29).

To confirm that induction of $\beta_3$ expression in our models is due to activation of the ERK pathway, L1-transfected 3T3 cells were treated with the inhibitor U0126 prior to FACS analysis. Consistent with a role for ERK, this inhibitor significantly decreased levels of $\beta_3$ expression after 72 h (Fig. 3K). Long-term treatment with U0126 was not observed to impact cell viability (data not shown). The association between ERK activation and induction of $\beta_3$ expression was further tested in 3T3 cells infected to express a $\beta_3$-RAF:ER fusion protein (30). The kinase activity of this fusion protein is selectively and conditionally activated by the addition of 4-hydroxystilbamidine (4HT) resulting in the selective and sustained activation of the ERK pathway within 10–15 min (30, 31). Treatment with 4HT and concomitant ERK activation in these 3T3 cells resulted in a marked increase in $\beta_3$ expression (Fig. 3L), and this induction could be blocked using the ERK pathway inhibitor U0126 (Fig. 3M). The increased $\alpha_6\beta_3$ expression in this model was only evident after 12–18 h of treatment with 4HT (Fig. 3N). Based on these results, we propose that L1 induces $\alpha_6\beta_3$ expression by virtue of inducing a sustained ERK response.

L1 Induces the Expression of Multiple ERK-regulated Gene Products—A cDNA microarray that allows the simultaneous detection of 96 genes intimately associated with motility and invasion (Mouse metastasis GE array Q series, SuperArray Inc.) was utilized to determine whether ectopic L1 expression induces more global changes in the expression of pro-invasive or pro-migratory gene products. A full listing of the genes on this array are available on the company website.

L1-dependent gene expression was assessed by comparing mRNA samples derived from mock- or L1-transfected 3T3 or K1735-C11 cells cultured under identical conditions. Based upon the observation that L1 can induce sustained ERK activation, it was further determined if L1-induced genes are also expressed as a result of conditional ERK activation. ERK-regulated genes were identified by comparing mRNA samples derived from 3T3/RAF:ER cells treated with 4HT or vehicle alone.

Fig. 4 lists multiple gene products that were significantly induced in 3T3 and K1735 cells both as a result of L1 expression and conditional ERK activation. Only those genes that were consistently induced in repeat experiments are listed. Notable examples include the cysteine proteases cathepsin-L and -B, the small GTPases RhoC and Rac-1, the matrix component osteopontin, and the adhesion receptor CD44 (Fig. 4). Although the distinct histological origin of 3T3 and K1735 cell lines, six of the nine genes identified in the 3T3 model were also confirmed in the K1735 model (Fig. 4).

Although ERK activation can account for the majority of the L1-induced gene products, this may not be the only mechanism by which L1 can induce changes in gene expression. Thus, several additional genes were induced as a result of L1 expression which were not significantly induced after conditional activation of the ERK pathway. In the 3T3 model, these genes include colony-stimulating factor-1, cystatin-C, thrombospondin-2, TIMP-2, and S100A4 (data not shown). Induction of colony-stimulating factor-1 and cystatin-C was also confirmed in the K1735 cells (data not shown). Although the microarray used can detect up to 96 different genes, it may only be useful for assessing genes expressed at high levels and, therefore, could result in an underestimation of the number of genes induced by L1. The $\beta_3$-integrin subunit, for example, is represented on the array, but no significant hybridization signal could be detected for this gene. Together, these data show that L1 can induce global changes in gene expression, and this can be attributed in large part to activation of the ERK pathway.

2Available on the World Wide Web at www.superarray.com/cancer.
ERK-regulated Gene Expression Contributes Directly to L1-mediated Motility—Several of the L1- and ERK-regulated gene products identified in this study, including integrin αvβ3 (32) and the GTPase rac-1 (33), have been strongly linked to motility. Studies were performed to determine whether induction of these gene products by L1 contributes directly to the expression of a motile phenotype.

To determine whether L1 induces a motile phenotype, we compared the migration of mock- or L1-transfected 3T3 cells to a variety of extracellular matrix components. Confirming that...
L1 and ERK-regulated Gene Expression

Fig. 5. Integrin α,β and the GTPase Rac-1 contribute to L1-dependent motility. A, migration of mock- or L1-transfected 3T3 cells toward vitronectin (6 h), native and denatured type I collagen (6 h), laminin (18 h), and fibronectin (4 h). B and C, migration of mock- or L1-transfected 3T3 cells to vitronectin or denatured collagen (type I) in the presence or absence of an antibody to α,β (2C9.G2, 40 μg/ml). D, migration of transfected K1735-C11 cells toward denatured collagen (type IV, 18 h) in the presence or absence of an antibody to α,β (2C9.G2, 40 μg/ml). E, migration of L1-transfected 3T3 cells to vitronectin was assessed after transient transfection with the dominant-negative Rac-1 construct Rac-N17. Control cells (Mock) received empty vector. A β-galactosidase reporter vector was used to identify transfected cells, and the migration of transfected 3T3 cells to vitronectin was assessed in a 6-h assay. F, Western blot analysis of mock- or L1-transfected 3T3 lysates using anti-Rac-1 mAb Cl.102. Actin levels were determined to confirm equal loading. G, 3T3ΔRAF:ER cells were pretreated with 4HT (15 ng/ml) or vehicle control (Me2SO) for 2 h prior to washing and the addition of 4HT (50 ng/ml) for a further 18 h. Migration of pretreated cells to denatured collagen was then assessed in a 6-h assay.

L1 induces cell motility, ectopic L1 expression in 3T3 cells was found to markedly enhance migration to a variety of substrates, including vitronectin, denatured type I collagen, laminin-1, and fibronectin (Fig. 5A). Consistent with a prior report (22), L1 was not observed to increase migration to native collagen.

A function-blocking antibody to the β3-integrin subunit was used to determine whether increased expression of integrin α,β contributes to the L1-dependent haptotaxis observed. Using this antibody, it was confirmed that the enhanced 3T3 cell migration to both vitronectin and denatured collagen can be attributed to increased or de novo utilization of integrin α,β3 (Fig. 5, B and C). Induction of α,β3-mediated haptotaxis was also confirmed after ectopic L1-expression in the K1735-C11 melanoma line (Fig. 5D). The α,β3-mediated migration observed was not significantly inhibited by the addition of a variety of anti-L1 monoclonal or polyclonal antibodies to the migration assays (data not shown). This finding suggests that direct L1 participation may not be required and that increased expression of α,β3 suffices to induce the migration observed. The enhanced L1-dependent migration to laminin and fibronectin (Fig. 5A) was inhibited using antibodies to α,β3 and α,β3 respectively (data not shown). The contribution of these integrins to L1-mediated haptotaxis is consistent with prior reports (21, 22).

Rac-1 has been shown to be an important signaling intermediate in cell motility (33), and this GTPase was induced 3-fold after ectopic L1 expression in 3T3 cells (Fig. 4). To determine whether rac-1 contributes to L1-dependent motility, L1-transfected 3T3 cells were transiently transfected with a vector encoding dominant-negative rac (Rac-N17) (24). Control cells received empty vector.

Expression of Rac-N17 did not have a significant impact on 3T3 adhesion or viability (data not shown) but did significantly reduce L1-dependent migration to vitronectin (Fig. 5E). In confirmation of the gene array data, increased levels of rac-1 protein were detected in lysates of L1-transfected 3T3 cells (Fig. 5F).

Our findings suggest that the induction of ERK-regulated gene products (e.g. α,β3 and rac-1) by L1 contributes to the induction of 3T3 cell motility. To confirm that ERK-dependent gene transcription suffices to promote 3T3 migration, we assessed the impact of conditional ERK activation and gene transcription on the migration of 3T3ΔRAF:ER cells (30, 31). After inducing ERK-activation with 4HT (18 h pretreatment) we observed a 1.8–2-fold induction of migration to denatured collagen (Fig. 5G). Induction of this migration was blocked both by the ERK pathway inhibitor U0126 and by the transcriptional inhibitor actinomycin-D (Fig. 5, G and H). At the concentration used (0.5 μg/ml for 2 h), actinomycin-D was not observed to affect 3T3 viability (data not shown). In time-course studies, it was observed that ERK activation had to be sustained for at least 12 h to induce 3T3 motility (data not shown). Based on these observations, we conclude that sustained ERK activation and concomitant gene transcription is
both necessary and sufficient to induce 3T3 migration.

**Induction of Cathepsin Activity by L1 Contributes to Matrix Invasion**—Cathepsins-L and -B have both been implicated in matrix invasion (34), and these cysteine proteases were strongly induced as a result of L1 expression in both 3T3 and K1735 cells (Fig. 4). To determine whether L1 induces an invasive phenotype, we utilized Transwell inserts coated with a barrier of reconstituted basement membrane (RBM) (matrigel, BD Biosciences). PDGF was added to lower chambers to serve as a chemoattractant and to activate L1-mediated motility. A comparison of RBM invasion by mock- or L1-transfected 3T3 cells confirmed the induction of an invasive phenotype with ectopic L1 expression (Fig. 6, A and B).

Cell permeable peptide inhibitors of cathepsins-L and B were used to determine whether these cysteine proteases contribute to the L1-mediated invasion observed. Peptide CA-064 Me (selective inhibitor of procathepsin-B) and peptide E-64-d (inhibitor of cathepsins-L and -B) both significantly inhibited invasion by L1-transfected 3T3 cells (Fig. 6C). At the concentrations used, neither peptide was observed to significantly affect 3T3 viability, and neither peptide significantly inhibited migration to RBM (data not shown). Induction of cathepsin expression and activity as a result of L1 expression was further confirmed using Magic Red fluorogenic substrates specific for cathepsins-L or B (Immunochemistry Technologies). Intracellular fluorescence, generated as a result of substrate cleavage, was assessed by flow cytometry, and this approach confirmed significantly higher levels of cathepsin-L and -B activity in the L1-transfectants (Fig. 6, D and E). Underscoring the importance of L1-mediated ERK activation, the expression and activity of both enzymes was significantly reduced after pretreating L1-transfected 3T3 cells with the ERK pathway inhibitor U0126 (Fig. 6, D and E, insets).

**DISCUSSION**

Prior reports (21, 22) have documented the potentiation of integrin-mediated haptotaxis after ectopic L1 expression and have provided evidence for non-translational mechanisms involving direct L1-integrin ligation and the induction of transient integrin signaling and endocytosis. In this study, we provide evidence that L1 can induce both motility and invasion via an additional novel mechanism that involves sustained ERK activation and the concomitant induction of motility-associated gene products. L1 expression, in two histologically distinct cell lines, is shown to induce higher constitutive levels of ERK activation and the concomitant expression of gene products that contribute directly to increased L1-dependent motility or invasion. Induction of the integrin $\alpha_\beta_3$ and the small GTPase rac-1 is shown to facilitate L1-dependent haptotaxis, whereas induction of cathepsin-L and -B promote matrix invasion. The ability of L1 to promote sustained ERK activation is shown to be critically dependent upon cell density and upon co-operation with serum or serum growth factors.

Although prior reports (35, 36) have shown that L1 cross-linking induces transient ERK activation, this is the first report that L1 can induce sustained ERK activation under normal culture conditions. This is an important distinction because sustained, rather than transient, activation of this pathway is often required for optimal gene transcription (28, 37). In this regard, we found that induction of $\alpha_\beta_3$ expression in 3T3 cells was only evident after ERK activation had been maintained for at least 12 h. Consistent with our observations, many of the L1- and ERK-induced genes identified in this study have previously been shown to be regulated by the ERK pathway, including cathepsins-L and -B, osteopontin, RhoC, CD44, and $\alpha_\beta_3$ (28, 38–40). Induction of ERK-dependent gene transcription by L1 has not been documented previously. However, other members of the immunoglobulin superfamily have been shown to induce ERK-dependent gene transcription. Neural cell adhesion molecule, for example, has been shown to promote neuritogenesis and gene transcription by inducing the sequential activation of ERK-1/2 and the transcription factor cAMP-response element-binding protein (41). Interestingly, induction of cAMP-response element-binding protein-dependent gene expression is known to require sustained ERK activation (42).
and neural cell adhesion molecule has been shown to support sustained ERK activation in both hippocampal neurons and PC12 cells (43).

Using 3T3RAF:ER cells (30), we observed that sustained ERK activation and gene transcription is sufficient to induce 3T3 cell motility to denatured collagen. Accordingly, L1 may induce migration via a translational mechanism without direct L1-integrin interaction and signaling (21, 22). However, L1 was also found to promote αβ3-dependent migration to fibronectin as described (21, 22), and we found no evidence that gene transcription is sufficient for this response. Thus, αβ3 levels were only minimally affected by L1 expression, and conditional ERK activation in 3T3RAF:ER failed to induce significant migration to fibronectin (data not shown). These findings suggest that both translational and non-translational mechanisms potentiate L1-mediated migration, depending upon which integrin or substrate is engaged.

The novel observation that L1 promotes the expression and utilization of integrin αβ3 has broad mechanistic ramifications for the participation of L1 in a variety of normal and pathological processes. Together with L1, this integrin has been shown to promote neurite extension (44–46), extravasation (16, 47, 48), myelination (49, 50), and metastasis (17, 32). In addition, L1 has recently been shown to promote fibroblast-matrix interactions in vitro, a process that could also involve αβ3 (9). Accordingly, a wide variety of L1-driven processes could involve the increased expression and utilization of αβ3. It is also noteworthy that L1 can also serve as a direct ligand for αβ3 (6), and that direct L1–αβ3 pairing has been shown to influence both neurite extension and extravasation (16, 44).

The contribution of L1 to normal central nervous system development could also be influenced by the transcription and expression of cathepsins-B and -L and rac-1. Cathepsins-B and -L are known to be important intermediates in L1-mediated processes, and neural cell adhesion molecule has been shown to support neurite extension and extravasation (16, 44).

Our novel finding that L1 induces matrix (RBM) invasion could account for reports documenting a strong association between L1 expression and metastasis of many types of tumor (38, 57, 58). In addition, it is noteworthy that L1 can also serve as a direct ligand for αβ3 (6), and that direct L1–αβ3 pairing has been shown to influence both neurite extension and extravasation (16, 44).

Sustained L1-dependent ERK activation in our 3T3 model required growth factor co-operation, and it is unclear whether this is due to a convergence of L1 and growth factor signaling pathways or whether L1 can directly or indirectly impact the activity of growth factor receptors. In support of the latter, it has recently been shown that L1-type molecules are able to interact directly with the EGF receptor to induce tyrosine kinase activity (59). It is also conceivable that L1 can influence growth factor receptor signaling by serving as a cellular ligand for integrins (21, 22). Thus, integrin ligation and clustering is known to result in the recruitment and activation of select growth factor receptors (60). Induction of αβ3 expression may also be significant because this integrin is known to collaborate directly with PDGF and its receptor to promote ERK activation (61).

This report provides the first evidence that L1 can induce global changes in gene expression resulting in a more motile or invasive phenotype. Such alterations in gene expression may be particularly important for stabilizing or maintaining changes in cellular phenotype that occur during normal development and tumorigenesis.

Acknowledgments—We thank Thomas Kaido for comments on this manuscript and Lilian Meyer for assistance with the FACS analysis.

REFERENCES

1. Brummendorf, T., and Rathjen, F. G. (1993) J. Neurochem. 61, 1207–1219
2. Richert, M. (1999) Neuron 17, 587–599
3. Lindner, J., Rathjen, F. G., and Schachner, M. (1983) Nature 305, 427–430
4. Thor, G., Prometeus, R., and Schachner, M. (1987) EMBO J. 6, 2581–2586
5. Ebeling, O., Durzlal, A., Aigner, S., Geiger, C., Schollhammer, S., Kemshead, J. T., Moller, P., Schwartz-Albiez, R., and Altevogt, P. (1996) Eur. J. Immu-

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
Biochem. Biophys. 396, 133–137
40. Zuber, J., Tchernitsa, O. I., Hinzmann, B., Schmitz, A. C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schafer, R. (2000) Nat. Genet. 2, 144–152
41. Jessen, U., Novitskaya, V., Pedersen, N., Serup, P., Berezin, V., and Bock, E. (2001) J. Neurochem. 79, 1149–1160
42. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) Cell 87, 1203–1214
43. Kolkova, K., Novitskaya, V., Pedersen, N., Berezin, V., and Bock, E. (2000) J. Neurosci. 20, 2238–2246
44. Yip, P. M., Zhao, X., Montgomery, A. M., and Siu, C.-H. (1998) Mol. Biol. Cell 9, 277–280
45. Ivins, J. K., Yurchenco, P. D., and Lander, A. D. (2000) J. Neurosci. 17, 6551–6560
46. Altetsee, C., Mullen, L., Kim, D., Pak, K., Brors, D., Dazert, S., and Ryan, A. F. (2001) Audiol. Neurootol. 2, 57–69
47. Hangan, D., Morris, V. L., Boeters, L., von Ballestem, C., Uniyal, S., and Chan, B. M. (1997) Cancer Res. 57, 3812–3817
48. Thompson, R. D., Wake, M. W., Larbi, K. Y., Dewar, A., Asimakopoulos, G., Nourshargh, S., and Contractor, C. (2000) J. Immunol. 165, 426–434
49. Bolvas, J. B., Setru, A., Baren, W., Buttery, P. C., LaFlamme, S. E., Franklin, R. J., and French-Constant, C. (2001) Curr. Biol. 6, 1445–1455
50. Bartsch, U. (2003) Front. Biosci. 8, D477–D490
51. Bednarzski, E., Ribak, C. E., and Lynch, G. (1997) J. Neurosci. 17, 4096–4101
52. Feilier, U., Kessler, B., Mothes, W., Goebel, H. H., Ploegh, H. L., Brunn, R. T., and Bjorn, R. O. (2002) Proc. Natl. Acad. Sci. U. S. A 99, 7883–7888
53. Meyer, G., and Feldman, E. L. (2002) J. Neurochem. 83, 490–503
54. Albeda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M., and Bok, C. A. (1991) Cancer Res. 51, 6757–6764
55. Hsu, M. Y., Shih, D. T., Meier, F. E., Van Belle, P., Hsu, J. Y., Elder, D. E., Bok, C. A., and Herlyn, M. (1998) Am. J. Pathol. 153, 1435–1442
56. Peitzelere, B., Stromblad, S., von Schalscha, T. L., Mijtjans, F., Paulats, J., Montgomery, A. M. P., Chereza, D. A., and Brooks, C. P. (1999) Cancer Res. 59, 2724–2730
57. Webb, C. P., Van Aelst, A., Wigler, M. H., and Wouda, G. F. (1998) Proc. Natl. Acad. Sci. U. S. A 95, 8773–8777
58. Govindarajan, B., Bai, X., Cohen, C., Zhong, H., Kilroy, S., Louis, G., Moses, M., and Aruis, J. L. (2003) J. Biol. Chem. 278, 9790–9795
59. Islam, R., Kristiansen, L. V., Romani, S., Garcia-Alonso, L., and Hortsch, M. (2004) Mol. Biol. Cell 15, 2003–2012
60. Yamada, K. M., and Even-Ram, S. (2002) Nat. Cell Biol. 4, E75–76
61. Schaller, M., Vuori, K., and Ruoslahti, E. (1997) EMBO J. 16, 5600–5607
Extracellular Signal-regulated Kinase (ERK)-dependent Gene Expression Contributes to L1 Cell Adhesion Molecule-dependent Motility and Invasion
Steve Silletti, Mayra Yebra, Brandon Perez, Vincenzo Cirulli, Martin McMahon and Anthony M. P. Montgomery

J. Biol. Chem. 2004, 279:28880-28888.
doi: 10.1074/jbc.M404075200 originally published online May 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404075200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 61 references, 32 of which can be accessed free at http://www.jbc.org/content/279/28/28880.full.html#ref-list-1