Specific Residues within the α2 Integrin Subunit Cytoplasmic Domain Regulate Migration and Cell Cycle Progression via Distinct MAPK Pathways*

Paul A. Klekotka, Samuel A. Santoro, Haochuan Wang, and Mary M. Zutter‡
From the Washington University School of Medicine, St. Louis, Missouri 63110

Received for publication, March 2, 2001, and in revised form, June 15, 2001
Published, JBC Papers in Press, June 20, 2001, DOI 10.1074/jbc.M101921200

The α2 integrin subunit cytoplasmic domain is necessary for epidermal growth factor (EGF)-stimulated chemotactic migration and insulin-dependent entry into S-phase of mammary epithelial cells adherent to type I collagen. Truncation mutants revealed that the seven amino acids, KYEKMTK, in addition to the GFFKR motif were sufficient for these functions. Mutation of tyrosine 1134 to alanine inhibited the ability of the cells to phosphorylate p38 MAPK and to migrate in response to EGF but had only a modest effect on the ability of the cells to induce sustained phosphorylation of the ERK MAPK, to up-regulate cyclin E and cdk2 expression, and to enter S-phase when adherent to type I collagen. Conversely, mutation of the lysine 1136 inhibited the ability of the cells to increase cyclin E and cdk2 expression, to maintain long term phosphorylation of the ERK MAPK, and to enter S-phase but had no effect on the ability of the cells to phosphorylate the p38 MAPK or to migrate on type I collagen in response to EGF. Methionine 1137 was essential for both migration and entry into S-phase. Thus, distinctly different structural elements of the α2 integrin cytoplasmic domain are required to engage the signaling pathways leading to cell migration or cell cycle progression.

The integrin family of heterodimeric cell surface adhesion receptors not only mediates adhesion to the extracellular matrix and other cells, but also serves to integrate signals from the outside to the inside of the cell (1–5). Signals from growth factor receptors acting in combination with signals from integrins are necessary to regulate complex cellular processes such as migration and proliferation. Adhesion to the extracellular matrix via integrins is required for growth factors to induce robust activation of both the extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase mitogen-activated protein kinase (JNK) cascades, which leads to progression through the G1 phase of the cell cycle (6–11). During chemotactic migration integrin ligation not only provides the adhesion necessary for traction on the extracellular matrix but also activates intracellular signaling molecules such as Rac, Cdc42, and p38 MAPK (12, 13).

The α1β1 and α2β1 integrins are expressed on the surface of many cell types where they serve as receptors for collagen and/or laminins (14–17). Studies by several groups, including our own, have shown that although the αβ1 and αβ1 integrins have similarities in ligand binding, they mediate different functions and are not redundant adhesive receptors (9, 10, 18–23). Recent studies employing wild type, truncated, and chimeric α2 integrin subunits indicate that the unique phenotypic influences of the αβ1 integrin on cell proliferation and migration on collagenous substrates are mediated through the cytoplasmic domain of the α2 subunit (13, 24). These findings suggested that certain residues present in the α2 integrin cytoplasmic domain not present in the α1 cytoplasmic domain might be responsible for mediating the differing phenotypes.

To begin to understand these differences we have undertaken a mutagenesis study to identify the residues within the α2 integrin cytoplasmic domain that are required to mediate chemotactic migration in response to epidermal growth factor (EGF) and entry into the cell cycle on type I collagen matrices in response to insulin. We find that the seven amino acids following the conserved GFFKR sequence are sufficient to recapitulate the phenotype of the full-length α2 integrin cytoplasmic domain in mammary epithelial cells. Mutation of the methionine at position 1137 to alanine inhibited both chemotactic migration and entry into S-phase. Mutation of the tyrosine at position 1134 to alanine severely inhibited migration, whereas it had only a modest effect on the ability of the cells to enter S-phase in the presence of insulin when adherent to type I collagen. Mutation of the lysine at position 1136 to alanine severely inhibited the ability of the mammary epithelial cells to enter S-phase, whereas it had no effect on the ability of the cells to migrate in response to EGF on a type I collagen matrix. Furthermore, these results confirmed our previous findings that phosphorylation of p38 MAPK downstream of the α2 integrin cytoplasmic domain was required for EGF-stimulated chemotactic migration and that up-regulation of cyclin E and cyclin dependent kinase 2 (cdk2) was required for entry into S-phase.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-α2 integrin subunit antibody was purchased from Life Technologies, Inc. Polyclonal anti-cyclin E, cdk2, and actin antibodies were purchased from Santa Cruz Biotechnology. Anti-bromodeoxyuridine (BrdUrd) antibody was purchased from Caltag Laboratories. PD98059 and geneticin were from Calbiochem. Fibronecin, BrdUrd, and propidium iodide were from Sigma. Polyclonal anti-cyclin E, cdk2, and/or laminins (14–17). Studies by several groups, including our own, have shown that although the αβ1 and αβ1 integrins have similarities in ligand binding, they mediate different functions and are not redundant adhesive receptors (9, 10, 18–23). Recent studies employing wild type, truncated, and chimeric α2 integrin subunits indicate that the unique phenotypic influences of the αβ1 integrin on cell proliferation and migration on collagenous substrates are mediated through the cytoplasmic domain of the α2 subunit (13, 24). These findings suggested that certain residues present in the α2 integrin cytoplasmic domain not present in the α1 cytoplasmic domain might be responsible for mediating the differing phenotypes.

To begin to understand these differences we have undertaken a mutagenesis study to identify the residues within the α2 integrin cytoplasmic domain that are required to mediate chemotactic migration in response to epidermal growth factor (EGF) and entry into the cell cycle on type I collagen matrices in response to insulin. We find that the seven amino acids following the conserved GFFKR sequence are sufficient to recapitulate the phenotype of the full-length α2 integrin cytoplasmic domain in mammary epithelial cells. Mutation of the methionine at position 1137 to alanine inhibited both chemotactic migration and entry into S-phase. Mutation of the tyrosine at position 1134 to alanine severely inhibited migration, whereas it had only a modest effect on the ability of the cells to enter S-phase in the presence of insulin when adherent to type I collagen. Mutation of the lysine at position 1136 to alanine severely inhibited the ability of the mammary epithelial cells to enter S-phase, whereas it had no effect on the ability of the cells to migrate in response to EGF on a type I collagen matrix. Furthermore, these results confirmed our previous findings that phosphorylation of p38 MAPK downstream of the α2 integrin cytoplasmic domain was required for EGF-stimulated chemotactic migration and that up-regulation of cyclin E and cyclin dependent kinase 2 (cdk2) was required for entry into S-phase.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-α2 integrin subunit antibody was purchased from Life Technologies, Inc. Polyclonal anti-cyclin E, cdk2, and actin antibodies were purchased from Santa Cruz Biotechnology. Anti-bromodeoxyuridine (BrdUrd) antibody was purchased from Caltag Laboratories. PD98059 and geneticin were from Calbiochem. Fibronecin, BrdUrd, and propidium iodide were from Sigma. Polyclonal anti-phospho-p38 MAPK, anti-total p38 MAPK, anti-total ERK antibodies, and monoclonal anti-phospho-ERK antibodies were obtained from New England Biolabs. Type I collagen was from Collaborative Biomedical
Products. Anti-α2 integrin subunit antibody was purchased from Chemicon International, Inc. 

**Mutagenesis**—Construction of the X2C2, X2C1, and X2C0 constructs was described previously (22). The chimeric human integrin α chain X2C4 cDNA, in the expression vector pFneo, was a generous gift from Dr. M. Strong (University of California, San Francisco). The chimeric cDNA construct was subcloned into the expression vector pSRα (Andrey Shaw, Washington University School of Medicine, St. Louis, MO). The nomenclature for the α2 integrin cytoplasmic domain truncation constructs was adopted from the work of Kassner et al. (25). The 1146, 1139, 1135, 1139 Y1134A, 1139 Y1134F, 1139 K1136A, and 1139 M1137A constructs were created by PCR-directed mutagenesis. A 5′ oligonucleotide including the Bgl II restriction enzyme site at base pair 2873 of the human α2 integrin subunit (5′-gaattacccaagacgatc-3′) was used in the PCR. The 3′-oligonucleotides for each reaction were as follows: 1146 (5′-tct aga tta ctc aat ctc tgg), 1139 (5′-tct aga ctc get att tgg tca tct ttt c), 1135 (5′-get cta gac gtc aat tca ttt c), 1139 Y1134F (5′-tct aga ctc get att tgg tca tct ttt caa ttc att tgg tca tct ttt c), 1139 Y1134A (5′-tct aga ctc get att tgg tca tct ttt cag ctt ttc ttt tg), 1139 K1136A (5′-tct aga ctc get att tgg tca tct ttt cat att ttc ttt tg), and 1139 M1137A (5′-tct aga ctc get att tgg tca tct ttt cat att ttc). Each 3′-oligonucleotide contained a stop codon and a sequence encoding an XbaI restriction enzyme site for subcloning purposes. The resulting PCR product was cloned into the pCR 2.1 vector (Invitrogen) and sequenced. The correct product was cloned into the pSRα expression vector containing the X2C4 construct using the BglII and XbaI sites to remove the α2 integrin cytoplasmic domain. All final expression constructs were verified by sequencing.

**Cell Culture and Transfection**—The subclones of the murine NMuMG cell line were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and insulin (5 μg/ml) (Lilly). The transfected cell lines (X2C2, X2C1, X2C0, and control) were developed as described previously and maintained in DMEM with geneticin (850 μg/ml) (22). The 1146, 1139, 1135, 1139 Y1134A, 1139 Y1134F, 1139 K1136A, 1139 T1138A, and 1139 M1137A plasmids were transfected into the NMuMG-3 cell line using Lipofectin (Life Technologies, Inc.) per the manufacturer’s instructions. Clonal cell lines were selected in media containing geneticin (850 μg/ml). Cell surface expression of the α2 integrin extracellular domain was evaluated by flow cytometric analysis as described previously (22).

**DNA Synthesis Assay**—Transfected NMuMG cells were serum-starved in media containing 0.4% serum and insulin (5 μg/ml) for 48 h and then in DMEM alone for 24 h, removed from the flask with trypsin/EDTA, and replated onto type I collagen (25 μg/ml)–coated dishes in media consisting of DMEM with insulin (5 μg/ml). After 22 h of incubation BrdUrd (10 μM final concentration) was added for the final 2 h, and the percentage of cells incorporating BrdUrd was determined as described by Tlsty et al. (26). Preliminary experiments revealed that the highest percentage of cells entering S-phase between 22 and 24 h following adhesion. No difference was detected when the cells were incubated in the absence of BrdUrd for the entire 24-h period or for up to 30 h. In inhibition experiments the cells were preincubated in the presence or absence of PD98059 (50 μM) for 15 min, or PD98059 was added to adherent cells in media at the indicated time points.

**Cell Migration on Type I Collagen**—Cell migration assays were performed using a modification (15) of the protocol described previously (27). The number of cells migrating to the lower surface was determined by counting the number of cells in 10 random high power (×400) fields. Data presented represent the mean ± S.E. of at least three separate experiments. Statistical analyses were carried out by unpaired t tests using GraphPad Prism version 2.01.

**Immunoblot Analysis**—Cells were serum-starved as described above, plated onto type I collagen (25 μg/ml)–coated dishes, and lysed after defined lengths of time in lysis buffer (50 mM HEPES [pH 7.2], 250 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 40 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM o-vanadate, and 1 mM dithiothreitol). Total protein concentration was determined by the Pierce protein assay (Fisher). Equivalent amounts of protein lysate were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P transfer membrane (Fisher). Immunoblots were developed overnight with the appropriate dilution of primary antibody at 4 °C followed by secondary horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit antibody (Jackson Immunoresearch) for 1 h at room temperature. ECL chemiluminescence system (Amersham Pharmacia Biotech) was used for visualization.

**Data Analysis**—All results shown are from at least 3 independent experiments, and data are presented as the mean ± S.E. Multiple
serum-starved for 24 h and plated on the upper surface of a transwell on type I collagen.

Entry into S-phase and EGF-stimulated chemotactic migration

**FIG. 2.**

/H9251 2 cytoplasmic domain-dependent insulin-stimulated A, X2C2, X2C1, 1146, and 1139 transfectants were determined 24 h after the serum-starved cells were plated on type I collagen in DMEM plus insulin (5 μg/ml). Migration through the pores of the filter was either unstimulated (Control) or stimulated with EGF (10 ng/ml) (EGF). Cell migration proceeded for 5 h in a 5% CO₂ humidified chamber at 37 °C. The number of cells attached to the lower surface of the transwell filter was quantitated microscopically. Results are presented as the mean ± S.E. of at least three separate experiments. B, entry into S-phase by clonal cell lines of the X2C2, X2C1, 1146, and 1139 transfectants was determined 24 h after the serum-starved cells were plated on type I collagen in the presence of insulin (Fig. 2D). These results indicate that one or more of the four amino acids deleted to create the 1135 construct was essential for mediating the functions of the α₂ integrin cytoplasmic domain exhibited by the X2C2 and 1139 transfectants.

Comparison of the seven amino acids distal to the GFFKR motif in the human α₂ integrin cytoplasmic domain to those

of the α₂ integrin cytoplasmic domain were sufficient to support EGF-stimulated chemotactic migration as well as insulin-dependent entry into S-phase in mammary epithelial cells adherent to type I collagen. In addition, since the 1139 construct encoded a truncated α₂ integrin cytoplasmic domain that was one amino acid shorter than the α₁ integrin cytoplasmic domain, the different phenotypes supported by the α₁ and α₂ cytoplasmic domains fused to the α₂ integrin extracellular and transmembrane domains were a consequence of specific sequences and not a function of the length of the cytoplasmic domain per se.

We have shown previously that the α₂ integrin subunit with a cytoplasmic domain truncated after the GFFKR motif (X2C0) was not able to support either entry into S-phase in the presence of insulin or EGF-stimulated chemotactic migration on type I collagen (22, 13). Since the data in Fig. 2, A and B, demonstrated that the seven amino acids following the GFFKR motif were sufficient to replicate the phenotype of the full-length α₂ integrin cytoplasmic domain, we made an additional cytoplasmic domain truncation by deleting the carboxyl-terminal 17 amino acids of the α₂ integrin cytoplasmic domain. As shown in Fig. 1B, this deletion created a construct that encoded a cytoplasmic domain that consisted of the GFFKR motif followed by the three amino acids of the α₂ integrin cytoplasmic domain. The 1135 construct was transfected into the NMuMG-3 cell line, and clonal cell lines that expressed the α₂ integrin extracellular domain at levels comparable to the X2C2, 1146, and 1139 transfectants were established, as described above (data not shown). Clones of the 1135 transfectants adhered to and spread on type I collagen in a manner similar to the X2C2 transfectants (data not shown). As shown in Fig. 2C, the ability of the 1135 transfectants to migrate in response to a chemotactic gradient of EGF was significantly reduced as compared with either the X2C2 or 1139 transfectants. Deletion of the four amino acids from the 1139 construct to create the 1135 construct also abrogated the ability of the 1135 transfectants to enter S-phase when plated on type I collagen in the presence of insulin (Fig. 2D). These results indicate that one or more of the four amino acids deleted to create the 1135 construct was essential for mediating the functions of the α₂ integrin cytoplasmic domain exhibited by the X2C2 and 1139 transfectants.

Comparison of the seven amino acids distal to the GFFKR motif in the human α₂ integrin cytoplasmic domain to those
residues in the murine and bovine α₂ integrin cytoplasmic domains revealed that only three of the amino acids are conserved across all three species (Fig. 3A). The three conserved residues include the tyrosine at position 1134, the lysine at position 1136, and the methionine at position 1137. Therefore, each of these three residues was individually mutated to an alanine in the 1139 cytoplasmic tail to create the 1139 Y1134A, 1139 K1136A, and 1139 M1137A constructs, respectively (Fig. 3A). The tyrosine at position 1134 was also mutated to a phenylalanine (1139 Y1134F). Finally, although not conserved across all three species, the threonine at position 1138 of the human α₂ subunit was mutated to alanine. These constructs were transfected into the NMuMG-3 cell line, and stable cell lines were established and selected as described above (data not shown). Individual clones of all four transfectants adhered to and spread on type I collagen in a manner similar to the X2C2 transfectants (data not shown). As shown in Fig. 3E, mutation of the lysine at position 1136 had no effect on the ability of the 1139 K1136A transfectants to migrate in response to EGF on type I collagen matrices. Substitution of the tyrosine at position 1134 with a phenylalanine had no effect on the ability of the 1139 Y1134F transfectants to migrate. However, mutation of either the tyrosine at position 1134 to alanine or the methionine at position 1137 to alanine severely reduced the ability of both the 1139 Y1134A and 1139 M1137A transfectants to migrate in response to EGF. Mutation of the threonine at position 1138 to alanine did not have a significant effect on chemotactic migration. These findings established the essential nature of the methionine at position 1137 and an aromatic amino acid at position 1134 for the ability of the α₂ integrin cytoplasmic domain to support EGF-dependent chemotactic migration by mammary epithelial cells on type I collagen matrices.

Next, we evaluated the ability of the 1139 Y1134A, 1139 K1136A, and 1139 M1137A transfectants to enter S-phase in an insulin-dependent manner following adhesion to type I collagen. The data in Fig. 3C demonstrate that mutation of either the lysine at position 1136 or the methionine at position 1137 abrogated the ability of the 1139 K1136A and 1139 M1137A transfectants to enter S-phase on type I collagen in the presence of insulin. However, in contrast to its effect on chemotactic migration, mutation of the tyrosine at position 1134 to an alanine had only a modest effect on the ability of the 1139 Y1134A transfectants to enter S-phase in an insulin-dependent manner when adherent to type I collagen. Replacement of the tyrosine at position 1134 with phenylalanine had little to no effect on the ability of the 1139 Y1134F transfectants to enter S-phase in the presence of insulin. Mutation of the threonine residue at position 1138 to alanine was also without effect (data not shown). These data demonstrate that the methionine at position 1137 was required for both EGF-dependent chemotactic migration and insulin-dependent entry into S-phase, whereas the lysine at position 1136 was required for entry into S-phase but not for migration. Conversely, the tyrosine, or at least an aromatic amino acid, at position 1134 was required for migration but was not essential for supporting α₂ integrin cytoplasmic domain-dependent entry into S-phase in the presence of insulin in mammary epithelial cells expressing a truncated α₂ integrin cytoplasmic domain.

Progression through the G₁ phase of the cell cycle and into S-phase requires signals from both growth factor receptors and the extracellular matrix. Signals from growth factor receptors and integrins converge on several signaling pathways including the ERK MAPK pathway (7, 28–32). We were interested in determining if the ERK MAPK pathway was differentially activated in the transfectants that enter S-phase (the X2C2,
was present for the first 6 h of the experiment and then re-
through the G1 phase of the cell cycle (Fig. 5). We regulated the levels of cyclin E and cdk2 as they progressed to
hesion to collagen and were therefore unable to enter S-phase. 

phosphorylation of ERK required for entry into S-phase, the X2C1 transfectants induced a transient increase in cyclin E 
and 1139 Y1134A transfectants in the presence of insulin ini-
tially if sustained activation of the ERK MAPK pathway was 
required for entry into S-phase, we utilized a pharmacologic 
hibitor of MAPK kinase, PD98059 (33). As shown in Fig. 4B, 
icubation of the X2C2 transfectants with PD98059 for the 
entire 24 h of the experiment significantly inhibited the num-
ber of cells entering S-phase, with a p < 0.002. When PD98059 
was present for the first 6 h of the experiment and then re-
moved and fresh media added, the inhibitor had no effect on the 
ability of the X2C2 transfectants to enter S-phase. Conversely, 
if the inhibitor was added after the first 6 h of the experiment, the compound inhibited the ability of the X2C2 transfectants to enter S-phase just as if it had been present for the entire experiment (Fig. 4B). Immunoblot analysis revealed that PD98059 inhibited the phosphorylation of ERK and that when PD98059 was removed the X2C2 transfectants were able to induce moderate levels of ERK phosphorylation again. There-
fore, sustained phosphorylation (>6 h) of the ERK MAPK was 
required for mammary epithelial cells to enter S-phase follow-
ing adhesion to type I collagen via the α2β1 integrin. Whereas 
adhesion to type I collagen of the X2C2, 1139, 1139 Y1134F, 
and 1139 Y1134A transfectants in the presence of insulin ini-
tiated a signal transduction cascade that induced the sustained 
phosphorylation of ERK required for entry into S-phase, the 
X2C1, 1139 K1136A, and 1139 M1137A transfectants were unable to induce sustained phosphorylation of ERK upon ad-
hesion to collagen and were therefore unable to enter S-phase. 

Following adhesion to collagen the X2C2 transfectants up-
regulated the levels of cyclin E and cdk2 as they progressed 
through the G1 phase of the cell cycle (Fig. 5A). However, the 
X2C1 transfectants induced a transient increase in cyclin E 
levels but failed to up-regulate cdk2 following adhesion to type I collagen and were arrested in G1. To determine if the inability of the 1139 K1136A and 1139 M1137A transfectants to progress through G1 and into S-phase was due to a failure to induce cyclin E and/or cdk2 expression, the transfectants were serum-starved, plated on type I collagen in the presence of insulin, and lysed at defined time points. As shown in Fig. 5A, transfectants that were able to enter S-phase (X2C2, 1139, 1139 Y1134F, and 1139 Y1134A) up-regulated the expression of both cyclin E and cdk2 following adhesion to type I collagen in an insulin-dependent manner, as expected. The X2C1 transfectants failed to increase substantially the expression of cdk2 or induce sustained up-regulation of cyclin E (Fig. 5A). The 1139 K1136A transfectants induced cyclin E expression but not up-regulation of cdk2, and the 1139 M1137A transfectants failed to substantially induce expression of cdk2 or sustained up-regulation of cyclin E (Fig. 5A). These results suggested that α2 integrin cytoplasmic domain-dependent induction of cyclin E and cdk2 levels, and therefore entry into S-phase, following adhesion to type I collagen was dependent on the presence of a lysine at position 1136 and a methionine at position 1137. 

To determine if sustained activation of the ERK MAPK path-
way was necessary for the increased expression of cyclin E and cdk2, their levels were evaluated in the X2C2 transfectants treated with PD98059. The X2C2 transfectants were serum-starved as described, harvested, preincubated with PD98059 for 15 min, plated on type I collagen in the presence of insulin, and lysed at the indicated time points. The immunoblots in Fig. 5B demonstrated that inhibition of the ERK MAPK pathway abrogated the ability of the X2C2 transfectants to induce cyclin E and cdk2 expression following adhesion to type I collagen via the α2β1 integrin in the presence of insulin. These results demonstrate that sustained activation of the ERK MAPK path-
way was required to mediate up-regulation of cyclin E and cdk2 downstream of the α2 integrin cytoplasmic domain following adhesion to collagen in an insulin-dependent manner. 

Previous studies revealed that activation of the p38 MAPK pathway following ligation of the α2β1 integrin was required for EGF-stimulated chemotactic migration on type I collagen (13). The phosphorylation status of p38 MAPK was evaluated by immunoblot analysis of the α2 integrin cytoplasmic domain mutant transfectants to determine if p38 MAPK was phospho-
rylated following adhesion to type I collagen in transfectants that migrated on collagenous substrates (X2C2, 1139, 1139 Y1134F, and 1139 K1136A transfectants) but not in those that did not migrate (X2C1, 1139 Y1134A, and 1139 M1137A transfectants). The transfectants were serum-starved in a manner identical to that described for the chemotactic migration as-
says, plated on type I collagen in the presence and absence of EGF (10 ng/ml), and lysed at defined time points. Adhesion of the X2C2, 1139, 1139 Y1134F, and 1139 K1136A transfectants to type I collagen resulted in phosphorylation of p38 MAPK within 15 min (Fig. 6). Phosphorylation of p38 MAPK was maintained for over 1 h and returned to baseline after 3 h. EGF slightly augmented p38 MAPK phosphorylation at 15 min and 1 h. Adhesion to collagen by the X2C1, 1139 Y1134A, and 1139 M1137A transfectants stimulated only minimal p38 MAPK phosphorylation that returned to baseline levels after 1 h. The presence of EGF slightly increased phosphorylation of p38 MAPK at 15 min in the X2C2, 1139 Y1134A, and 1139 M1137A transfectants (Fig. 6). These results indicate that phosphoryl-
ation of the p38 MAPK pathway downstream of the α2 integrin cytoplasmic domain (13) was dependent upon the presence of a methionine at position 1137 and a hydrophobic residue at position 1134. 

DISCUSSION

The seven amino acids (KYEKMTK) following the GFFKR sequence of the α2 integrin cytoplasmic domain are sufficient to support both EGF-stimulated chemotactic migration and insulin-dependent entry into S-phase in mammary epithelial cells adherent to type I collagen. The methionine at position 1137 is required for both the sustained phosphorylation of the p38 MAPK leading to α2 cytoplasmic domain-dependent, EGF-stimulated migration and sustained phosphorylation of the ERK MAPK, leading to up-regulation of cyclin E and cdk2 levels, and entry into S-phase. In contrast the tyrosine, or at least an aromatic amino acid, at position 1134 is required for p38 MAPK-dependent, EGF-stimulated chemotactic migration but not for ERK MAPK-stimulated up-regulation of cyclin E and cdk2 and entry into S-phase. Conversely, the lysine at position 1136 is required for ERK-stimulated induction of cyclin E and cdk2 and insulin-dependent entry into S-phase but not for chemotactic migration.

Studies have demonstrated that although the ERK MAPK pathway is required for entry into S-phase following integrin ligation (7, 9–11, 34), its activation is not required for chemotactic migration on type I collagen via the α2β1 integrin (12, 13). The results with the 1139 Y1134A and 1139 K1136A transfectants support and extend these findings. The 1139 Y1134A transfectants stimulated sustained phosphorylation of
ERK MAPK and entry into S-phase but did not migrate. Whereas the 1139 K1136A transfectants were able to migrate in response to a chemotactic gradient of EGF, they were not able to induce sustained phosphorylation of ERK or to enter S-phase in the presence of insulin following adhesion to collagen.

Several molecules have been shown to interact with integrin α subunit cytoplasmic domains including calreticulin (35), F-actin (36), paxillin (37), Nischarin (38), Mss4, BIN1, and two novel cDNAs (39). Calreticulin has been shown to interact with the conserved GFFKR motif in the integrin α subunit cytoplasmic domain (35, 40), and it has been postulated to stabilize the high affinity state of the αβ2 integrin (41). This region is present in all of the constructs utilized in this study, and all of the transfectants adhered equally well to collagen. Therefore, it is unlikely that differential binding of calreticulin explains the differing phenotypes, although it cannot be ruled out. F-actin has been shown to bind to a portion of the α2 integrin subunit cytoplasmic domain that was deleted in the functional 1139 construct (36). It is thus unlikely that F-actin binding plays a role in controlling EGF-stimulated migration or insulin-dependent entry into S-phase. Although paxillin has been shown to interact specifically with the α2 integrin subunit cytoplasmic domain to facilitate cell migration, it does not appear to bind other α subunits (37). Recently, Nischarin has been reported to interact with the α2 integrin subunit and inhibit cell migration (38). Although paxillin and Nischarin may not interact directly with the α2 integrin cytoplasmic domain, the results of those studies provide evidence that cytoplasmic signaling molecules can bind directly to integrin α subunit cytoplasmic domains to regulate cellular phenotypes such as migration. Recently, Wixler et al. (39) have shown that the nucleotide exchange factor Mss4, the putative tumor suppressor protein BIN1, and two novel proteins interact differentially with different integrin α subunit cytoplasmic tails by yeast two-hybrid analysis. Their studies revealed that Mss4 interacts with the α2, but not the α1, subunit cytoplasmic domain and that the interaction of all four proteins with the α2 integrin subunit cytoplasmic domain is dependent in part on the juxtamembrane region of cytoplasmic domain, including the GFFKR motif (39). These results suggest that each α integrin subunit can bind different cytoplasmic signaling molecules necessary to activate multiple signal transduction cascades leading to changes in cellular migration and proliferation.

Two separate signaling pathways downstream of the α2 integrin cytoplasmic domain mediate chemotactic migration and entry into S-phase. Distinct residues of the cytoplasmic domain are responsible for initiating the different signals that lead to either cell migration or cell proliferation. How can two residues separated by only two amino acids control such differing phenotypes? The most likely explanation is that the two residues are responsible for interactions with different adaptor molecules. Secondary structure prediction indicates that the cytoplasmic domain of the α2 integrin subunit adopts an α-helical conformation across the amino acids FKRKYERM, which contains all of the residues mutated in this study (42, 44). Similar

![Diagram](http://www.jbc.org/)
The analysis of the cytoplasmic domain encoded by the 1139 construct revealed that it is also predicted to form an α-helix in this same region. Analysis of the mutations created in this study revealed that none of them are predicted to disrupt the helical nature of this region of the α2 cytoplasmic domain. Mapping the amino acids FKRKYEKM of the α2 cytoplasmic domain to a helical wheel revealed that the conserved basic residues (Lys-1131, Arg-1132, and Lys-1136) cluster on one face of the helix, and the conserved hydrophobic residues (Phe-1130, Tyr-1134, and Met-1137) are clustered on the other face (Fig. 7). This arrangement suggests a model to explain how mutations at position 1134 can affect migration while mutations at position 1136 can affect entry into S-phase; they are on opposite faces of the helix where they presumably interact with different adapter molecules.

The basic amino acids on one face of the helix result in a positive charge on that face that could mediate interactions with other cytoplasmic molecules. For example, the positively charged lysine at position 1136 may interact with residues in the α1 subunit cytoplasmic domain, possibly by forming a salt bridge with an acidic residue in the α2 integrin cytoplasmic domain. Hughes et al. (45) elegantly demonstrated that the arginine in the GFFKR motif of the αIIb integrin cytoplasmic domain formed a salt bridge with an aspartic acid in the α3 integrin cytoplasmic domain. The lysine at position 1136 maps to the same face of the α2-helix potentially formed by the α2 integrin cytoplasmic domain (45) and could form a salt bridge with the glutamic acid that is eight amino acids distal from the plasma membrane in the α1 integrin cytoplasmic domain. This interaction may be necessary to activate a signaling pathway downstream of the β1 integrin cytoplasmic domain that is required for entry into S-phase, but not for EGF-dependent chemotactic migration. Alternatively, basic amino acids have been shown to mediate the interaction of several cytoplasmic molecules with phosphatidylinositol 4,5-bisphosphate (46–48). The α2 integrin subunit cytoplasmic domain contains several basic amino acids, including the lysine at position 1136, and these residues may allow the integrin cytoplasmic domain to bind polyphosphoinositides or other cy-
toplastic signaling molecules. The K1136A mutation could disrupt the charged face of the helix and thus prevent any interactions with signaling molecules.

Arrangement of the $\alpha_2$ cytoplasmic domain in an $\alpha$-helical structure places the conserved hydrophobic residues (Tyr-1134 and Met-1137) on the face of the helix opposite to the charged residues, which could allow for interaction with a different molecule(s) on the hydrophobic face of the helix. The aromatic residue at position 1134 (Tyr or Phe) is probably responsible for interacting with another cytoplasmic signaling molecule that initiates a signal transduction cascade necessary for migration but not for entry into S-phase. The effectiveness of the phenylalanine substitution indicates that phosphorylation of Tyr-1134 to an alanine may disrupt this interaction directly or cause a conformational change that disrupts the necessary interaction. Mutation of the methionine at position 1137 to alanine may result in a mis-folded, or otherwise non-functional, interaction. Mutation of the methionine at position 1137 to alanine may disrupt this interaction directly or cause a conformational change that disrupts the necessary interaction. Mutation of the methionine at position 1137 to alanine may result in a mis-folded, or otherwise non-functional, interaction.

The $\alpha_2$ integrin cytoplasmic domain may hamper metastasis (51–54). Furthermore, Henriet et al. (43) have recently shown that melanoma cells, utilizing the $\alpha_2\beta_1$ integrin, growth-arrest on fibrillar collagen and proliferate on non-fibrillar collagen. We have shown herein that the lysine at position 1136 of the $\alpha_2$ cytoplasmic domain is necessary for mammary epithelial cell proliferation on non-fibrillar collagen. Therefore, a molecule that interferes with the interaction of Lys-1136 and its partner molecule could be potentially useful for inhibiting tumor cell proliferation.

In conclusion, we have shown that mutation of a single residue within a truncated, but fully functional, $\alpha_2$ integrin cytoplasmic domain can inhibit EGF-stimulated chemotactic migration but not insulin-dependent entry into S-phase, and vice versa, in mammary epithelial cells adherent to type I collagen. The essential residues likely mediate interactions with different adaptor or signaling molecules that activate separate signaling pathways such as the p38 and ERK MAPK pathways, respectively. This is the first study, to our knowledge, to demonstrate that single amino acid substitutions within an integrin $\alpha$ subunit cytoplasmic domain can differentially affect the activation of different MAPK pathways and dramatically influence cellular phenotypes.

Acknowledgments—We thank Dr. Martin E. Hemler for providing constructs used in these studies; Glenda White, Bruce Linders, and George Li for their technical expertise; and Mary Beth Flynn for expert secretarial assistance.

REFERENCES

1. Clark, K. A., and Brugge, J. S. (1995) Science 268, 233–239
2. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Biol. 11, 549–599
3. Yamada, K. M., and Miyamoto, S. (1995) Curr. Opin. Cell Biol. 7, 681–689
4. Howe, A., Aplin, A. E., Alahari, S. K., and Juliano, R. L. (1998) Curr. Opin. Cell Biol. 10, 229–231
5. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
6. Miyamoto, S., Teramoto, H., Coom, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) J. Cell Biol. 131, 791–805
7. Aplin, A. E., Short, S. M., and Juliano, R. L. (1999) J. Biol. Chem. 274, 31223–31229
8. Oktay, M., Warzy, K. K., Dans, M., Birge, R. B., and Giancotti, F. G. (1999) J. Cell Biol. 145, 1461–1469
9. Warzy, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) Curr. Opin. Cell Biol. 8, 783–793
10. Warzy, K. K., Mainiero, A., Zurzolo, C., and Giancotti, F. G. (1998) Cell 94, 625–634
11. Mainiero, F., Murgia, C., Warzy, K. K., Curatola, A. M., Pepe, A., Blumberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. (1997) EMBO J. 16, 2365–2375
12. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) Nature 390, 632–636
13. Kleekotka, P. A., Santoro, S. A., and Zutter, M. M. (2001) J. Biol. Chem. 276, 9503–9511
14. Hall, D. E., Reichardt, L. F., Crowdy, E., Holley, B., Moezzi, H., Sonnenberg, A., and Damsky, C. H. (1990) J. Cell Biol. 110, 2175–2184
15. Ignatiou, M. J., Large, T. H., Houde, M., Tawil, J. W., Barton, E., Esch, F., Carbonetto, S., and Reichardt, L. F. (1990) J. Cell Biol. 111, 759–772
Residues in α5 Cytoplasmic Domain Control Cell Phenotype

16. Santoro, S. A. (1986) Cell 46, 913–920
17. Staatz, W. D., Walsh, J. J., Pexton, T., Santoro, S. A. (1990) J. Biol. Chem. 265, 4778–4781
18. Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995) J. Cell Biol. 131, 1903–1915
19. Zutter, M. M., Santoro, S. A., Staatz, W. D., and Tsung, Y. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 92, 7417–7421
20. Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V. M., and Heino, J. (1996) EMBO J. 15, 6181–6189
21. Gotwals, P. J., Chi-Rosso, G., Lindner, V., Yang, J., Ling, L., Fawell, S. E., and Koteliansky, V. E. (1996) J. Clin. Invest. 97, 2469–2477
22. Zutter, M. M., Santoro, S. A., Wu, J. E., Wakatsuki, T., Dickeson, S. K., and Elson, E. L. (1999) J. Biol. Chem. 274, 927–940
23. Ivaska, J., Reunanen, H., Westermarck, J., Koivisto, L., Kahari, V. M., and Heino, J. (1999) J. Cell Biol. 147, 401–416
24. Klekotka, P. A., Santoro, S. A., Ho, A., Dowdy, S. F., and Zutter, M. M. (2001) Am. J. Pathol., in press
25. Kassner, P. D., Kawaguchi, S., and Hemler, M. E. (1994) J. Biol. Chem. 269, 19859–19867
26. Tseyt, T., Briot, A., and Poulou, B. (1995) Methods Enzymol. 254, 125–133
27. Santoro, S. A., Zutter, M. M., Staatz, W. D., Saelman, E. U. M., and Keely, P. J. (1995) Methods Enzymol. 245, 147–183
28. Renshaw, M. W., Ben, X., and Schwartz, M. A. (1997) EMBO J. 16, 5592–5599
29. Chen, Q., Lin, T. H., Iber, C. J., and Juliano, R. L. (1996) J. Biol. Chem. 271, 18122–18127
30. Bottazzi, M. E., and Asselin, R. K. (1997) Trends Cell Biol. 7, 348–352
31. Howe, A. K., and Juliano, R. L. (1998) J. Biol. Chem. 273, 27268–27274
32. Renshaw, M. W., Price, L. S., and Schwartz, M. A. (1999) J. Cell Biol. 147, 611–618
33. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
34. Lavoie, J., L’Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 20608–20616
35. Bojmani, M. V., Finlay, B. B., Gray, V., and Dedhar, S. (1991) Biochemistry 30, 9859–9866
36. Kieffer, J. D., Plopper, G., Ingber, D. E., Hartwig, J. H., and Kupper, T. S. (1988) Biochips. Res. Commun. 217, 466–474
37. Liu, S., Thomas, S. M., Woodside, D. G., Rose, D. M., Kiosses, W. B., Pfaff, M., and Ginsberg, M. H. (1999) Nature 402, 676–681
38. Alahari, S. K., Lee, J. W., and Juliano, R. L. (2000) J. Cell Biol. 151, 1141–1154
39. Wiens, V., Laplantine, E., Geerts, D., Sonnenberg, A., Petersohn, D., Eckes, B., Paulsson, M., and Aumailley, M. (1999) FEBS Lett. 445, 351–355
40. Leung-Hagesteijn, C. Y., Milankov, K., Michalak, M., Wilkins, J., and Dedha, R. S. (1994) J. Cell Sci. 107, 589–590
41. Coppolino, M., Leung-Hagesteijn, C., Dedhar, S., and Wilkins, J. (1995) J. Biol. Chem. 270, 23132–23138
42. O’Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Lofthus, J. C., Shattil, S. J., and Ginsberg, M. H. (1994) J. Cell Biol. 124, 1047–1059
43. Henriet, P., Zhung, Z. D., Brooks, P. C., Weinberg, K. I., and DeClerck, Y. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10026–10031
44. Deleage, G., Blanchet, C., and Guerjon, C. (1997) Biochimie (Paris) 79, 681–686
45. Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J. A., Shattil, S. J., and Ginsberg, M. H. (1996) J. Biol. Chem. 271, 6571–6574
46. Yu, F. X., Sun, H. Q., Janmey, P. A., and Yin, H. L. (1992) J. Biol. Chem. 267, 14616–14621
47. Lee, D., Oh, E., Woods, A., Couchman, J. R., and Lee, W. (1998) J. Biol. Chem. 273, 13022–13029
48. Barret, C., Roy, C., Montcourrier, P., Mangeat, P., and Niggli, V. (2000) J. Cell Biol. 151, 1067–1079
49. Werr, J., Johansson, J., Eriksson, E. E., Hedqvist, P., Russlahti, E., and Lindbom, L. (2000) Blood 95, 1804–1809
50. de Fougereoles, A. R., Sprague, A. G., Nickerson-Nutter, C. L., Chi-Rosso, G., Bennert, P. D., Gardner, H., Gotwals, P. J., Lob, B. R., and Koteliansky, V. E. (2000) J. Clin. Invest. 105, 721–729
51. Lundstrom, A., Holmblom, J., Lindqvist, C., and Nordstrom, T. (1998) Biochem. Biophys. Res. Commun. 250, 725–731
52. Fishman, D. A., Kears, A., Chilukuri, K., Bafetti, L. M., O’Toole, E. A., Georgacopoulos, J., Ravosa, M. J., and Stack, M. S. (1998) Invasion Metastasis 18, 15–26
53. Lang, S. H., Clarke, N. W., George, N. J., and Testa, N. G. (1997) Clin. Exp. Metastasis 15, 218–227
54. Sato, M., Narita, T., Kawakami-Kimura, N., Higashiyama, S., Taniguchi, N., Altews, S., Hachimato, M., Manabe, T., and Kannagi, R. (1996) Cancer Lett. 102, 183–189
