Characterization of Downstream Ras Signals That Induce Alternative Protease-dependent Invasive Phenotypes*

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Simone Silberman‡, Mark Janulis§, and Richard M. Schultz§

From the ‡Department of Pathology and the §Department of Molecular and Cellular Biochemistry, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

Invasive and metastatic cells require protease expression for migration through the extracellular matrix. Metastatic NIH 3T3 fibroblasts transformed by different activated ras genes showed two different protease phenotypes, ras

\[ uPA + CL \] and ras

\[ CL + uPA \] (Zhang, J-Y., and Schultz, R. M. (1992) Cancer Research 52, 6682–6689). Phenotype ras

\[ uPA + CL \] is dependent on expression of the serine-type protease urokinase plasminogen activator (uPA) and the phenotype ras

\[ CL + uPA \] on the cysteine-type protease cathepsin L (CL) for lung colonization in experimental metastasis. The existence of multiple invasive phenotypes on ras-isofrom transformation implied the activation of alternative pathways downstream from Ras. We now show that c-Raf-1, extracellular signal-regulated protein kinase (ERK)-1, and ERK-2 are hyperphosphorylated, and the ERK activity is high in both the uPA- and CL-dependent ras-transformed invasive phenotypes. Levels of c-Jun and c-Jun NH2-terminal kinase (JNK) activity are also high in the uPA-dependent phenotype, but they are almost undetectable in the CL-dependent phenotype. The uPA Ras-response element is a PEA3/URTF element, and mobility shift assays show a strong PEA3/URTF protein band in the uPA-dependent phenotype. This band is competed by a consensus AP-1 DNA sequence and by antibodies to PEA3 and c-Jun. Thus, the uPA invasive phenotype appears to require the activation of Ras/PEA3 and c-Jun transcription factors activated by the ERK and JNK pathways, while the CL-invasive phenotype appears to require ERK activity with suppression of JNK and c-Jun activities. These postulates are supported by the induction of a dominant negative c-Jun, TAM67, into cells expressing ras

\[ uPA + CL \] and ras

\[ CL + uPA \]. We conclude that the JNK pathway acts as a switch between two distinct protease phenotypes that are redundant in their abilities to grow tumors and metastasize.

Increased protease expressions are correlated with the invasive properties of migrating cells. The proteases are required for the degradation of the ECM through which cells must invade during the course of their invasions and migrations. Proteases that are expressed and secreted by invading cells include urokinase plasminogen activator, the cathepsins B, D, and L, and the matrix metalloproteinases (MMPs), including interstitial collagenase (MMP-1), the 72- and 92-kDa type IV collagenases (MMP-2 and MMP-9), stromelysins 1 and 3 (MMP-3, MMP-11), and matrilysin (MMP-7) (1–7). Since any one migrating cell only expresses or induces a limited spectrum of these proteases, redundant protease mechanisms must exist for cellular migration through ECM under both normal and pathological conditions. Furthermore, switching between redundant mechanisms may be a common event. For example, prior data have shown a dependence on plasminogen activator activity in the cellular invasions that occur during fertilization, embryogenesis, angiogenesis, neuronal development, and macrophage migration (1, 2, 4, 5, 8–10). However, only slight perturbations in these processes are observed in plasminogen activator-deficient mice (4, 8), suggesting that readily available compensatory protease mechanisms exist. The pathways that regulate these protease expression patterns and, therefore, the mechanisms responsible for the compensatory switching between protease-induction pathways are essentially unknown.

In a previous report, we showed two distinct ras-transformed metastatic phenotypes in NIH 3T3 fibroblasts, based on differences in expression of either uPA or CL and the corresponding ability of either uPA or CL to facilitate lung colonization in nude mice (11). The NIH 3T3 cells transformed by the EJ/vHa-ras gene are an example of phenotype ras

\[ uPA + CL \]. These cells showed a high constitutive expression of uPA, low constitutive expression of CL, and uPA-dependent (CL-independent) lung colonization in nude mice. In contrast, NIH 3T3 cells transformed by the RAS1Lae-deleted gene were of phenotype ras

\[ CL + uPA \]. These cells showed high constitutive expression of CL, low constitutive expression of uPA, and CL-dependent (uPA-independent) lung colonization (11).

A role for uPA in invasion and metastasis has been demonstrated by experiments in which metastasis was inhibited by anti-uPA antibodies and by the expression of uPA sense or antisense CDNAs in transformed cells that either promoted or inhibited, respectively, tumor cell invasion and metastasis (12–14). Pro-uPA may be secreted by migrating or stromal cells in the environment of migrating cells and may be activated while bound to specific uPA receptors expressed on the cell surface of invading cells (15). uPA promotes the degradation of ECM...
proteins either acting alone or through activation of plasminogen to plasmin (6), which, in turn, may initiate an extracellular cascade leading to the activation of the pro-metalloproteinases (6, 16, 17). A possible role for CR1 in invasion and metastasis is supported by reports of a correlation between a high CR1 expression and high levels of invasion or metastasis in particular tumor types (reviewed by Gottesman and co-workers (7)). CR1 is secreted by transformed cells in the pro-form and is processed extracellularly to an enzymatically active form (18–20). CR1 has been shown to act on types I and IV collagen and other major components of the ECM (21–23).

Multiple pathways, in addition to the c-Raf → ERK pathway, have recently been shown to be activated by Ras (24, 25). Ras may thus be a switching molecule that interprets multiple cellular physiological signals to control such diverse physiological processes as proliferation and differentiation (26) through selective activation of downstream pathways. Different Ras isoforms may discriminate between downstream pathways through structurally-dependent differences in affinities for downstream effector protein molecules (27), resulting in an affinity-dependent selection among the possible downstream effectors and their corresponding pathways.

This paper will provide evidence that the Ras → ERK and Ras → JNK pathways are required for the ras<sup>αPA</sup>/Cl<sup>-</sup> phenotype; while in the ras<sup>αCL</sup>/αPA<sup>-</sup> phenotype, the ERK pathway is activated, but the JNK pathway leading to the activation of c-Jun is inhibited. These data support a hypothesis that the JNK pathway regulates a switch between two different transformed phenotypes that carry out metastasis by different protease mechanisms.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture Conditions**—NIH 3T3 cells and NIH 3T3 cells containing the EJvHa-ras and Ras<sup>αCLI</sup>del oncogene constructs were originally obtained from Merck Sharp & Dohme Research Laboratories (West Point, PA) and cultured at low passage number as described previously by Bradley et al. (28). The characteristics and metastatic potential of these transformed cells have been previously reported by Bradley et al. (28) and by Zhang and Schultz (11). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO<sub>2</sub> atmosphere as described previously (11).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared by the method of Digman et al. (29) from NIH 3T3 (control), EJvHa-ras, and Ras<sup>αCLI</sup>del cells. The cells (1 × 10<sup>6</sup>) were washed with cold PBS and suspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, and 0.2 mM in freshly added phenylmethylsulfon fluoride). The swollen cells were homogenized, and the nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM phenylmethylsulfon fluoride). A high salt buffer (with 2 mM KCl in place of 20 mM KCl) was added dropwise to release soluble proteins. The solution was centrifuged, the supernatant was dialyzed, and the protein concentrations in the extracts were determined by a Bio-Rad protein kit and normalized for protein concentration, and the samples were boiled in SDS-PAGE sample buffer and resolved on a 12% SDS-PAGE gel. The gels were transferred and analyzed by autoradiography with quantification by densitometry (Bio-Imaging analyzer).

**JNK In-gel gel Assay**—The procedure was similar to that previously described by Gotob et al. (30). Cells were plated as in the Western blot analysis procedure and lysed with RIP buffer, the lysates were normalized for protein concentration, and the samples were boiled in the SDS-PAGE sample buffer. Approximately 50 µg of protein were loaded per lane in an SDS-PAGE 12% polyacrylamide gel in which myelin basic protein (Sigma) was incorporated at a concentration of 0.4 mg/ml. After electrophoresis, the gel was washed twice with 1 M sodium orthovanadate. The immune complexes were collected by centrifugation for 30 min at 2500 rpm in a microcentrifuge and washed 4 times with 1 ml of RIP buffer. Samples were boiled in SDS-PAGE sample buffer and resolved on a 12% SDS-PAGE gel. The gels were transferred and analyzed by autoradiography with quantification by densitometry (Bio-Imaging analyzer).

**JNK Assay**—The plasmid pGEX-GST-c-Jun-1–135 synthesized by J. R. Woodgett (31) was obtained from J.M. Kyriakis (Massachusetts General Hospital) and used to transform Escherichia coli. The GST-c-Jun substrate for JNK was isolated as described by Frangioni and Neel (32). GST-c-Jun was attached to hexylglutathione-agarose beads (Sigma) and used to assay JNK as described by Westwick and Brenner (33). The cells were lysates incubated with the GST-c-Jun-agarose were obtained from NIH 3T3 cells and ras-transformed NIH 3T3 cells grown in 100 mm plates, were washed with ice-cold PBS, and were treated with cell lysis buffer (20 mM HEPES, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT and 0.3% Nonidet P-40). After 30 min at 4 °C, the plates were scraped and the lysate clarified by centrifugation at 14,000 rpm for 10 min. The supernatant was assayed for protein
concentration by the Bio-Rad protein kit, normalized for protein concentration, and stored in aliquots at −80 °C. For the kinase assay, 80 μg protein aliquots were incubated with 1 μg of GST-c-Jun bound to glutathione-agarose beads for 3 h at 4 °C. The kinase assay was carried out as described by Westwick and Brenner (33), and the 32P-c-Jun-GST product was analyzed in a 12% SDS-polyacrylamide gel. The gel was dried and exposed overnight for autoradiography with quantification by densitometry.

Northern Analysis for uPA and CL—The mouse CL cDNA in pUC-19 was a gift from G. Gary Sahagian (Tufts University School of Medicine, MA), and the mouse uPA cDNA plasmid pDB4501 (a mouse uPA Partner III cDNA fragment in pSP64) was a gift of Dominique Belin (Centre Medical Universitaire, Switzerland). The CL mRNA 32P-labeled hybridization probe was produced from the 766-base pair EcoRI fragment of the pUC19-CL by random priming (Ready-to-Go Kit, Pharmacia Biotech Inc.). The 32P-labeled uPA mRNA antisense hybridization probe was prepared using SP6 RNA polymerase (Life Technologies, Inc.) from the SP6 promoter site in the pDB4501 plasmid.

Generation and Characterization of EJ/vHa-ras-transformed Cells Containing a Dominant Negative c-Jun (TAM67)—The plasmid pCMV-TAM67 containing the dominant negative c-Jun, TAM67, and the control plasmid pCMV (empty vector) were a generous gift from Michael J. Birrer (NCI, MD). TAM67 is a deletion mutant of the c-Jun gene that lacks the N-terminal transactivation domain (amino acids 3–128) (34, 35). Cells were co-transfected with pcDNA3 (Invitrogen, CA) along with pCMV-TAM67 or pCMV (empty vector) using the calcium phosphate method (36). In brief, cells were grown to a concentration of 3 × 104 cells/100-mm dish, the media was replaced with 5 ml of serum-free DMEM, and incubated 2 h. Cells were then exposed in DNA/calcium phosphate solution (2 μg of pCMV-TAM67 or of pCMV-empty vector along with 1 μg of pcDNA3 and made up to 10 μg of DNA with salmon sperm DNA) for 4 h, were rinsed with PBS, and were grown overnight in 10% FBS-DMEM. The following day, cells were divided into three 100-mm plates, and the media was changed to 10% FBS-DMEM containing 200 μg/ml G418. After 48 h, the concentration of G418 was increased to 600 μg/ml, and the cells were selected for 14 to 21 days. G418-resistant colonies were isolated for expansion using cloning cylinders, and cells were maintained in 10% FBS-DMEM containing 200 μg/ml G418 until analysis.

The persistence of the transformed phenotype in the TAM67 expressing cells was shown by the ability of the cells to form colonies in soft agar, utilizing the procedure of Cox and Der (37).

RESULTS

Several parallel pathways are known to exist downstream from Ras (24, 25, 38). A well characterized downstream pathway is from Ras → c-Raf-1 → MEK → ERK-1/ERK-2 (Fig. 1). The phosphorylation of transcription factors by the ERKs activate the expression of genes that are believed to cause new DNA synthesis and cell division (24, 38). A parallel kinase cascade pathway from Ras utilizes the Ras-like p21 GTP binding proteins Rac and CDC42, leading to the activation of JNK and p38 kinase. Activation of JNK leads to the phosphorylation and activation of the transcription factors c-Jun and ATF-2 (24, 39–43). This pathway is also activated in a Ras-independent manner by cytokines and other stress factors (39–43). In this work, we have compared the signal transduction pathway from Ras to ERK and Ras to JNK in EJ/vHa-ras-transformed (phenotype ras1Leu+/uPA+/CL−), and untransformed NIH 3T3 fibroblasts to determine the Ras downstream signals that may regulate the switching between the uPA-dependent and CL-dependent invasion mechanisms.

Assay of the Raf → ERK Pathway Activity in ras-transformed and Untransformed Fibroblasts—The relative concentrations of c-Raf-1 and the MAP kinases ERK-1 and ERK-2 were determined by Western blot analysis (Fig. 2). The levels of c-Raf-1, ERK1, and ERK2 were not significantly different between the untransformed NIH 3T3 cells and the EJ/vHa-ras- or Ras1Leu+del-transformed NIH 3T3 cells. Therefore, differences between the untransformed and Ras-transformed phenotypes are not due to underlying differences in the concentrations of these downstream Ras signal transduction proteins.

Activation of protein molecules downstream from Ras in the Raf → MEK → ERK kinase cascade occurs on their phosphorylation by the preceding kinase in the cascade (44–46). To assay the phosphorylation state of c-Raf-1 and c-ERK-1/2, cells were grown in [32P]phosphate for 4 h prior to lysis and immunoprecipitation with c-Raf-1 or c-ERK-1 specific antibodies. The polyclonal ERK-1 antibody cross-reacts with ERK-2, and both antibodies are immunoprecipitated. [32P]-c-Raf-1 was at a 2- to 3-fold higher concentration in both EJ/vHa-ras- and Ras1Leu+del-transformed cells than in untransformed NIH 3T3 cells (Fig. 3A, Table I). Levels of [32P]-ERK-1 and [32P]-ERK-2 were 9- to 13-fold higher in the EJ/vHa-ras-transformed cells than in untransformed NIH 3T3 cells (Fig. 3B and Table I). In the Ras1Leu+del-transformed cells, the [32P]-ERK-1 and [32P]-ERK-2 levels were 6- to 7-fold higher than in NIH 3T3 cells. The difference in [32P]ERK levels between EJ/vHa-ras-transformed cells and Ras1Leu+del-transformed cells do not appear functionally significant since we observed similar ERK catalytic rates (see below) in both these ras-transformed phenotypes.

ERK activity was measured directly by an in-gel substrate assay with the ERK specific substrate myelin basic protein (33, 47). [32P] Incorporation into myelin basic protein was observed at a position corresponding to the molecular weights of the ERKs (Fig. 3C). ERK activities were significantly higher in the EJ/vHa-ras- and Ras1Leu+del-transformed cell lines (lanes 2 and 3,
anti-ERK-1 antibody (SC-93) which cross-reacts with ERK-2. anti-c-Raf antibody (SC-227); 3.4 ± 0.3-fold and 3.0 ± 0.4-fold, respectively) than for NIH 3T3 controls (Fig. 3C, lane 1). Thus, the high ERK catalytic activities and the hyperphosphorylation of c-Raf-1 and ERK-1/ERK-2 in the EJ/vHa- and RAS1Leu-del-transformed cells show that the c-Raf-1 → ERK pathway is constitutively activated to approximately an equal extent in both the uPA- and CL-dependent ras-transformed phenotypes relative to NIH 3T3 controls.

Assay of JNK and the c-Jun Transcription Factor in ras-transformed and Untransformed Fibroblasts—Western blot analysis of cell lysates for c-Fos levels showed that levels of c-Fos were equal in the untransformed NIH 3T3 and EJ/vHa-ras- and RAS1Leu-del-transformed cells (Fig. 4A). However, Western blot analysis of c-Jun showed a relatively high concentration of c-Jun in the EJ/vHa-ras-transformed cells (phenotype ras<sup>uPA<sub>/CL</sub>-</sup>), intermediate amounts of c-Jun in untransformed NIH 3T3 control cells, and low concentrations of c-Jun in RAS1Leu-del-transformed cells (phenotype ras<sup>CL<sub>/uPA</sub>-</sup>) (Fig. 4B).

Treatment of cells with [32P]phosphate following immunoprecipitation of the cell lysates with c-Jun antibody, SDS-polyacrylamide electrophoresis of the immunoprecipitate, and autoradiography to visualize 32P-labeled proteins showed two major bands of molecular weights characteristic of both 32P-c-Jun and 32P-c-Fos (Fig. 5B). Western blot analysis of the immunoprecipitate with anti-Fos antibody showed the band at 61 kDa to be a Fos antigen (Fig. 5C). The 32P-c-Jun and 32P-c-Fos bands were present at high relative concentrations in the EJ/vHa-ras-transformed cells, intermediate levels in untransformed NIH 3T3 cells, and near or below our limits of detection in the RAS1Leu-del-transformed cells (Fig. 5B and Table I). An assay of JNK was made directly against a substrate GST-Jun–1–135, which specifically assays JNK but not ERK (33). Fig. 5A shows a 3-fold higher level of 32P-GST-Jun in EJ/vHa-ras-transformed cells than in untransformed NIH 3T3 cells, while in RAS1Leu-del cells (phenotype ras<sup>CL<sub>/uPA</sub>-</sup>), JNK activity is barely observable (~20% of the level in NIH 3T3 controls).

The lower levels of JNK catalytic activity and 32P-c-Jun in the RAS1Leu-del-transformed cells suggests that the JNK activity may be inhibited in the CL-dependent phenotype. Western blot analysis for JNK protein showed similar concentrations in RAS1Leu-del-transformed cells and NIH 3T3 cells (data not shown), demonstrating that the low activity found for JNK in RAS1Leu-del cells is not due to a decrease in JNK protein levels in these cells but to a lower enzymatic activity of the JNK protein.

**Analysis by Mobility Shift Assays of the PEA3/AP-1-like uPA Ras-response Element**—The mouse and human uPA genes have been sequenced and several functional enhancer elements in their promoter regions identified (48–50). Lengyl et al. (51) showed that the Ras response element of the uPA promoter is a PEA3/AP-1-like element located upstream of the start of transcription between –1973 and –1960 in the human uPA gene. Nerlov et al. (52) had previously shown the same sequence acts as an uPA enhancer in transformed human HepG2.
and HT 1080 cells. In the mouse uPA promoter, the homologous element is located ~2.4 kb upstream from the start of transcription (50). The element is similar to the PEA3/AP-1 element identified as a Ras-response element in the collagenase and stromelysin protease genes by Waasylk and co-workers (53) and Waasylk et al. (54). However, the consensus AP-1 sequence, TGA(T/C)A, is a heptamer, and the AP-1-like sequence of the PEA3/AP-1-like element of the uPA promoter is an octomer sequence (TGAGG(T/C)A in mouse (50) and TGAaGTA(C/A) in human (48)). These octomer sequences of the uPA promoter are more similar to a consensus octomer CRE enhancer element, TGACGTCA, than to AP-1. Because of the similarity to both the consensus AP-1 and CRE, this sequence has been designated URTF (urokinase transcription factor family) rather than AP-1-like by Rørth et al. (50). In agreement with the nomenclature of Rørth, we refer to this AP-1/CRE-like element as the URTF element.

Fig. 6 shows gel retardation assays with a 32P-doublestranded DNA sequence containing the PEA3/URTF enhancer sequence treated with nuclear extracts from EJ/vHa-ras, Ras1Leu-del, and untransformed NIH 3T3 cells. The gel shifts show two bands (bands 1 and 2) corresponding to protein-DNA complexes of faster and slower mobilities that are competed by unlabeled PEA3/URTF. The slower mobility complex (band 1) is present in high concentrations in the EJ/vHa-ras-transformed cells, intermediate concentrations in untransformed NIH 3T3 cells, and low concentrations in Ras1Leu-del-transformed cells (Fig. 6). The variation in concentration of band 1 among the three cell lines positively correlates with the previously reported levels of uPA mRNA and uPA gene transcription rates among the three cell lines (11).

Fig. 7A shows gel retardation experiments with the doublestranded deoxyligonucleotide sequences, given in Table II, in EJ/vHa-ras- and Ras1Leu-del-transformed cells. The gel retardation of the AP-1 consensus sequence gives an intense broad band with the nuclear extract of EJ/vHa-ras-transformed cells and a less broad and less intense band with Ras1Leu-del-transformed cells (Fig. 7A, lanes 1 and 6). A complex is not formed with the mutant AP-1 sequence (lanes 2 and 7). The broadness of the AP-1 complex in the gel retardation experiments may be due to the presence of multiple members of the Jun, Fos, and CREB/ATF families that can associate with the consensus AP-1 enhancer element. A similar broad AP-1 band has been previously observed in other cell types and shown to be due to the binding of multiple AP-1 proteins to the AP-1 enhancer element (55). The narrower and less intense band observed for the AP-1 complex with the Ras1Leu-del-transformed cells indicates the presence of lower concentrations and/or less diversity of AP-1 binding proteins in these cells. In addition to the broad AP-1 band, the Ras1Leu-del-transformed cells also show a slower migrating AP-1 DNA-protein complex band with identical mobility to band 2 observed with the PEA3/URTF sequence (lane 6). While the consensus AP-1 forms a broad protein-DNA complex band, the mutant AP-1 sequence formed no strong protein-DNA complexes (lanes 2 and 7). The URTF element shows a complex with overlapping mobility to the complexes observed with the consensus AP-1 in both EJ/vHa-ras- and Ras1Leu-del-transformed cells (lanes 3 and 8). However, the URTF bands are not as intense or broad as that seen for the consensus AP-1 sequence.

The mutant PEA3/URTF sequence showed no retarded complex with the nuclear extract of EJ/vHa-ras-transformed cells (Fig. 7A, lane 4). However in the Ras1Leu-del-transformed cells, a complex with mobility identical to that of the band 2 complex is observed with the mutant PEA3/URTF sequence (Fig. 7A,
formed, RAS1Leudel-transformed, and NIH 3T3 cells. The expression of the TAM67 gene in G418-resistant clones was shown by Northern analysis to migrate at 868 base pairs, while native c-Jun mRNA migrates at approximately 2.7–3.2 kilobase pairs. Of 11 clones analyzed, 5 were positive for TAM67 mRNA expression. The levels of CL mRNA and uPA mRNA were then determined in all TAM67 mRNA-expressing clones. Fig. 9 shows the results for controls transfected with pCMV-empty vector and for all 5 of the EJ/vHa-ras-TAM67 clones isolated. In cells transfected with pCMV-empty vector, all G418-resistant clones tested maintained their expected phenotype. Also as expected, RAS1Leudel and NIH 3T3 cells, which normally contain low constitutive levels of c-Jun (Fig. 4), show no changes in protease phenotype on their transfec- tion with TAM67 (data not shown). However, TAM67-expressing EJ/vHa-ras cells showed a conversion in levels of uPA and CL mRNAs from those of a rasuPA+/CL− phenotype to levels characteristic of RAS1Leudel-transformed cells of phenotype rasCL+/uPA−. These data suggest that activation of c-Jun is necessary for the rasuPA+/CL− phenotype and inhibition of c-Jun activity generates a switch to the rasCL+/uPA− phenotype.

**DISCUSSION**

Previous data from our laboratory demonstrated that two distinct metastatic phenotypes were generated in NIH 3T3 cells transfomed by EJ/vHa-ras and RAS1Leudel genes (11). One phenotype, designated rasuPA+/CL− and represented by the EJ/vHa-ras-transformed NIH 3T3 cells, shows high constitutive levels of uPA mRNA and low constitutive CL mRNA levels. Experiments utilizing uPA and CL antisense oligonucleotides to selectively suppress uPA or CL gene expressions demonstrated that lung colonization by EJ/vHa-ras-transformed cells was uPA-dependent and CL-independent. In contrast, the second ras-transformed phenotype, designated rasCL+/uPA− and represented by RAS1Leudel-transformed NIH 3T3 cells, shows high constitutive levels of CL mRNA and low constitutive levels of uPA mRNA. Antisense oligonucleotide suppression experiments demonstrated that lung colonization of RAS1Leudel-transformed cells was CL-dependent and uPA-independent. Both these cell lines were found to give a similar large number of tumors in nude mice lung colonization assays (11). This report characterizes the signal transduction pathways downstream from mutant Ras for the two redundant metastatic ras-transformed phenotypes that utilize either uPA or CL to achieve experimental metastasis. Metastasis is an ECM invasive process that requires proteases to degrade the proteins of the ECM to allow the movement of migrating cells through the ECM (6).

Fig. 1 displays pathways downstream from Ras that lead to the activation of gene transcription factors. The pathway through c-Raf-1 → MAPK kinase/MEK → MAPK/ERK-1/ERK-2 leads to activation by phosphorylation of transcription factors of the Ets family and Myc (59–61). Early studies indicated that the ERK1 and/or the ERK2 may also activate c-Jun and c-Fos (62, 63). While some evidence exists for activation of c-Jun and c-Fos by ERK (40, 62, 63), phosphorylation of c-Jun by ERK may also inhibit c-Jun mediated trans-activations (40). Accordingly, whether activation of c-Jun occurs by ERK phosphorylation is not clear. However, c-Jun and c-Fos activation does occur on phosphorylation by the c-Jun and c-Fos specific kinases, JNK and FRK, that are distinct from the MAPKs ERK-1 and ERK-2. The activation of JNK and FRK occur via a Ras-dependent pathway not involving c-Raf-1, MEK, or ERK (31, 38–43) (Fig. 1).

The concentrations of c-Raf-1, ERK-1, and ERK-2 in the Ras → ERK pathway were determined by Western blot analysis and shown to be at approximately equal concentrations in the

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**Fig. 6. Electrophoretic mobility shift assays using nuclear extracts from NIH 3T3, EJ/vHa-ras- and RAS1Leudel-transformed cells, and labeled 32P-PEA3/URTF enhancer.** A, arrows point to slow (band 1, upper) and fast (band 2, lower) mobility complexes (see text). B, electrophoretic mobility shift assays in EJ/vHa-ras-transformed cells with the 32P-PEA3/URTF sequence and the competition for the complex by unlabeled PEA3/URTF. Arrow shows band 1 that positively correlates with uPA gene expression (see text). The lane labeled NS shows the lack of competition by a nonspecific control oligo that contains an NFkB element sequence and is present at a 200-fold molar excess (lane 5).

Lane 9). A similar mutant PEA3/URTF has been shown to be unresponsive to Ras stimulation in a chloramphenicol acetyltransferase enhancer assay system (51), suggesting that the band 2 complex formed between mutant-PEA3/URTF and nuclear proteins in RAS1Leudel-transformed cells is non-functional.

Fig. 1B shows the competition between 32P-labeled sequences and unlabeled sequences for proteins in gel retardation. In these experiments, only the nuclear extracts of the EJ/vHa-ras-transformed cells were utilized. The experiment shows the broad complex formed with the consensus 32P-AP-1 sequence is completely competed by a 100-fold excess unlabeled AP-1. The 32P-URTF sequence is competed by both the unlabeled URTF sequence and by the unlabeled consensus AP-1 sequence, indicating an overlap of binding specificity between the consensus AP-1 and URTF sequences. The 32P-PEA3/URTF complex (band 1) is efficiently competed by the unlabeled consensus AP-1 sequence. This competition further indicates an overlap of specificity between the consensus AP-1 sequence and the URTF sequence of the mouse uPA PEA3/URTF enhancer.

Fig. 8 shows the inhibition of the PEA3/URTF band 1 by both a PEA3 antibody and an antibody against c-Jun, indicating the presence of both PEA3 and c-Jun specific antigens in the band 1 complex. The inhibition of complex formation by anti-PEA3 and anti-c-Jun specific antibodies is in agreement with previously reported experiments showing that Ets transcription factors and c-Jun trans-activate through the PEA3/AP-1-like element (48, 50–52, 56–58).

**Conversion of the rasuPA+/CL− Phenotype to the rasCL+/uPA− Phenotype by Introduction of a Dominant Negative c-Jun into EJ/vHa-ras-transformed Cells—A dominant negative c-Jun, TAM67, missing the c-Jun N-terminal trans-activating domain (amino acids 3–122) was transfected into the EJ/vHa-ras-transformed.
EJ/vHa-ras-transformed cells, the RAS1Leul-del-transformed cells, and the untransformed NIH 3T3 cells (Fig. 2). Accordingly, differences in the transformed phenotypes are not due to underlying differences in the cellular concentrations of these proteins. However, the \[^{32}P\]phosphate-labeled forms of c-Raf-1, ERK-1, and ERK-2 were present at high concentrations only in the ras-transformed cells (Fig. 3, A and B, and Table I). Direct kinase assays confirm the high activity of MAPK/ERK in both ras-transformed phenotypes (Fig. 3C). Therefore, the c-Raf-1 \(\rightarrow\) ERK pathway may be required for transformation by both mutant Ras isoforms. This agrees with evidence from other laboratories in which transfection of NIH 3T3 cells with constitutively active forms of Raf or MEK lead to transformation. Conversely, experiments in which the Raf \(\rightarrow\) ERK pathway is inhibited by dominant negative c-Raf-1, ERK, or phosphatase deactivators of ERK have been shown to lead to reversion of Ras-induced transformation (26, 64–66).

In contrast to the observation of ERK activation in both ras-transformed phenotypes, the c-Jun activating kinase, JNK,
suggests that the transformed phenotype in several c-Jun expression has been shown to cause reversion of the shown to partially revert K-ras-transformed NIH 3T3 cells to a normal phenotype (68, 69). However, other data have shown that rat embryo fibroblasts, unable to express c-Jun, could be transformed into a tumorigenic and metastatic phenotype by an activated ras gene (70). Our data show in the RAS1\textsuperscript{Lew}-del-transformed cells low concentrations of c-Jun and phosphorylated-c-Jun, corresponding with an inhibition of JNK activity and indicating that a c-Jun-independent transformation pathway exists in the RAS1\textsuperscript{Lew}-del-transformed cell line. In addition, the inhibition of c-Jun activity with TAM67 in EJ/vHa-ras- and RAS1\textsuperscript{Lew}-del-transformed cells results in the maintenance of the transformed state as assayed by colony formation in soft agar (data not shown), supporting the existence of c-Jun-independent transformed phenotypes.

The Ras-response element in the uPA promoter has been shown in ras-transformed human OVCAR cells to be the PEA3/URTF sequence (51). The mouse uPA promoter contains an upstream PEA3/URTF enhancer sequence identical to the human sequence, except for a single G → A change in the URTF site (see Table II). A PEA3/URTF enhancer has also been found in the porcine uPA gene promoter and has an identical sequence to that of mouse (57). Mutational studies of the PEA3/URTF sequence in chloramphenicol acetyltransferase expression vectors with native and mutant human PEA3/URTF sequences have shown that both the PEA3 and URTF elements are required for enhancer stimulation by Ras (51). The PEA3 element binds members of the Ets family of transcription factors which are activated upon stimulation of the Raf/ERK pathway (61). The URTF element in the human PEA3/URTF sequence appears to be trans-activated in ras-transformed human OVCAR cells by c-Jun as a dominant negative c-Jun, TAM67, inhibits PEA3/URTF enhancer stimulation (51). Further evidence that c-Jun activates the URTF enhancer comes from experiments in porcine LCC-PK1 cells in which okadaic acid inhibition of the JNK phosphatase increased the activity of JNK, the corresponding concentration of 32P-c-Jun, and the enhancer activity of the URTF element in the PEA3/URTF sequence (57). The immunological characterization of the URTF gel retardation complex with human HepG2 cell nuclear extract by monospecific antibodies showed the complex to be a heterodimer of c-Jun and ATF-2 (58). It has been suggested that ATF-2 is activated by the same pathway as c-Jun (43) (Fig. 1). Activation of the Ras-response element in the uPA promoter thus appears to require the activation of an Ets family transcription factor through stimulation of the c-Raf-1 → ERK-1/ERK-2 pathway (51, 61, 68, 71) and of c-Jun and perhaps ATF-2 through activation by the JNK pathway (43, 51, 57, 58).

In this work, we show by gel shift analysis that the intensity of one of the retarded bands (band 1) positively correlates with uPA mRNA levels and the rate of uPA expression among the cell lines (Figs. 6 and 7). Other bands were shown to be nonspecific since they appear in the presence of a mutant URTF sequence and/or with the consensus AP-1 alone (Fig. 7). Further analysis of band 1 supports the AP-1-like character of the octomer URTF mouse sequence in the PEA3/URTF element.

The URTF gel retardation band was shown to be competed by a consensus AP-1, and the consensus AP-1 also competed for ATF-2 through activation by the JNK pathway (43) (Fig. 1). Activation of the Ras-response element in the uPA promoter thus appears to require the activation of an Ets family transcription factor through stimulation of the c-Raf-1 → ERK-1/ERK-2 pathway (51, 61, 68, 71) and of c-Jun and perhaps ATF-2 through activation by the JNK pathway (43, 51, 57, 58).

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The URTF gel retardation band was shown to be competed by a consensus AP-1, and the consensus AP-1 also competed for the band 1 proteins of the complete PEA3/URTF (Fig. 7). Furthermore, an antibody to the AP-1 transcription factor, c-Jun, prevented formation of the PEA3/URTF gel retardation band (Fig. 8). An antibody to transcription factor PE3, part of the PEA3 subfamily of the Ets family of transcription factors, also inhibited the formation of the PEA3/URTF band 1 complex (Fig. 8). PEA3 proteins are a subfamily of the Ets family of transcription factors. Recently discovered members of the Ets family of transcription factors that may regulate uPA through the PEA3 element include ERM, a ubiquitous member of the PEA3 subfamily, and the Ets transcription factor E1A-F, which has been shown to up-regulate the stromelysin, type I collagen-
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ase, and 92-kDa type IV collagenase genes (72, 73).

Therefore, the simplest explanations for our data are that:

(1) in the untransformed NIH 3T3 cells, neither the ERK or JNK signaling pathways are active, leading to low levels of active Ets and c-Jun/ATF-2 and low constitutive expression of uPA and CL. (2) In the EJ/vHa-ras cells, both the Ras → ERK and the Ras → JNK pathways are active, and their combined activities are required to activate the PEA3 and c-Jun transactivating proteins on the uPA PEA3/URTF enhancer. (3) In the RAS1-leu-del-transformed cells, the activated Ets transcription factors are present since the Ras → ERK pathway is active, but JNK activity is absent and therefore insufficient levels of active c-Jun exist to activate the uPA PEA3/URTF enhancer. We have shown a switch in phenotype from ras-uPA+/CL- to ras-uPA+/CL+ by the selective inhibition of c-Jun with TAM67 in EJ/vHa-ras cells. Thus, the presence of active Ets and an absence of active c-Jun lead to increased expression of CL by an unknown mechanism. We cannot at present eliminate the possibility that other pathways downstream of Ras are active in the EJ/vHa-ras- and RAS1-leu-del-transformed cells.

Only recently have the details of redundant pathways begun to be explored as greater knowledge of the similarities and differences of functionally homologous protein families become available. We have shown that in ras-transformed NIH 3T3 cells, redundant transformed phenotypes exist that differ in both signaling pathway activations and genes expressed. While we have studied only changes in the regulation of uPA and CL gene expressions, we expect that the differential activation of pathways we observe affect a whole spectrum of gene expressions.

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