Gene Trapping Identifies Inhibitors of Oncogenic Transformation

THE TISSUE INHIBITOR OF METALLOPROTEINASES-3 (TIMP3) AND COLLAGEN TYPE I α2 (COL1A2) ARE EPIDERMAL GROWTH FACTOR-REGULATED GROWTH REPRESSORS

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A gene trap strategy has been used to identify genes that are repressed in cells transformed by an activated epidermal growth factor (EGF)/EGF receptor signal transduction pathway. EGF receptor-expressing NIH3T3 cells (HER1 cells) were infected with a retrovirus containing coding sequences for the human CD2 antigen and for secreted alkaline phosphatase in the U3 region. By selecting for and against CD2 expression, we obtained clones in which the gene trap had integrated into genes selectively repressed by EGF. Two of these clones encoded for the secreted extracellular matrix proteins TIMP3 and COL1A2. We show here that both genes are downstream targets of RAS and are specifically repressed by EGF-induced transformation. Moreover, this strategy tags tumor suppressor genes in their normal chromosomal location, thereby improving target-specific screens for antineoplastic drugs.

Gene fusions between promoterless and/or enhancerless genes encoding easily assayable gene products with the controlling elements of cellular genes have provided valuable tools for studying gene function and regulation in prokaryotic and eukaryotic cells. Vectors developed for use in mammalian cells, referred to as “gene traps,” insert a reporter gene into mostly random chromosomal sites, including transcriptionally active regions. By selecting for gene expression, recombinants are obtained in which the reporter gene is fused to the regulatory elements of endogenous genes. Transcripts generated by these fusions faithfully reflect the activity of the tagged cellular gene and thus provide an effective means to study the expression of genes in their normal chromosomal location. Moreover, gene traps are highly mutagenic both in vitro and in vivo and provide molecular tags to clone any gene whose function is linked to an observable phenotype (1–9).

Gene entrapment strategies have been successfully used to induce mutations in both cultured cells and transgenic mice. By using appropriate reporter systems, we and others (10–13) have identified genes that are either induced or repressed during important biological processes such as cellular differentiation or programmed cell death. To extend these observations, we sought to determine whether gene trapping would enable the recovery of genes with growth inhibitory and/or tumor suppressing functions. The basic assumption was that fusion transcripts induced between the selectable marker gene of a gene trap and the cellular sequences of a putative tumor suppressor gene would be repressed by an oncogenic signal. Such events can then be selected for by using a marker gene which allows selection for and against expression. Moreover, reporter genes expressed from the promoters of putative tumor suppressor genes should provide natural molecular targets for drug screening assays, thereby improving discovery of new anti-neoplastic drugs.

To test the suitability of this approach, a conditional transformation system was chosen that is based on a reversible activation of the epidermal growth factor receptor (EGFR/HER1) signal transduction pathway. EGFR belongs to the HER family of tyrosine kinase receptors that have been implicated in the development and progression of several types of human cancer (14, 15). When overexpressed in NIH3T3 cells, EGFR transforms in a ligand-dependent manner and thus functions as an oncogene.

A gene trap retrovirus vector carrying coding sequences for the human CD2 cell surface receptor and for secreted human placental alkaline phosphatase (SEAP) in its U3 region was used to infect a subclone (HER1KA1) of an EGFR-expressing NIH3T3 cell line (16, 17). This subclone is highly and reversibly transformable by EGF. Selection for and against CD2 expression yielded clones in which the cell-provirus fusion transcripts were repressed by EGF-mediated transformation. Analysis of upstream proviral flanking sequences revealed that in two instances the gene trap integration had disrupted known genes. These genes code for the tissue inhibitor of metalloproteinases 3 (Timp3) (18) and for procollagen type α2 (Col1A2) (19).

We show here that Timp3 and Col1A2 are downstream targets of c-RAS within the EGF/EGF signal transduction pathway. Both genes inhibit oncogenic transformation and are specifically repressed in EGF-transformed cells.

MATERIALS AND METHODS

Plasmids and Viruses—The pGgU3CD2SEAPen− (−) plasmid was derived from pGgU3HisTkNeo− (−) (2) by replacing the His coding se-

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PBS, phosphate-buffered saline; SEAP, secreted alkaline phosphatase; TIMP, tissue inhibitor of metalloproteinases; COL1A2, collagen type α2; DMEM, Dulbecco’s modified Eagle’s medium; RACE, rapid amplification of cDNA ends; IRES, internal ribosomal entry site; LTR, long terminal repeat; kb, kilobase pair(s); SRBC, sheep red blood cells; Ate, anhydrotetracycline.

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quences in the U3 region of the 3'-LTR with CD2-IRE5-SEAP. CD2 sequences were recovered from pPH3CD2 (20). IRES-SEAP sequences were recovered from pSBc2-SEAP (21). pGUG3CD2-SEAPen (-) was transfected into GP + E86 producer cells as described previously (2). A clonal cell line producing 1 × 10^5 Neo^R colony-forming units/ml was isolated and used to infect HER1KA1 cells at a multiplicity of infection = 3.

Tim3 sequences obtained from BSTim3^TM (NMI, San Diego, CA) were ligated as EcoRI fragments into the corresponding sites of the pBabe puromycin. pBabe Tim3 was transfected into BOSC23 cells and virus containing supernatants were used to infect HER1KA1 and HER1-6.1 cells as described previously (2, 30).

The human Ha-RAS^Leu61 coding sequences were recovered from pZIPNeoSVH-ras-leu61 (23) and cloned into the EcoRI/HindIII sites of the expression vector pTBC1 downstream of a tetO7/CMV promoter (24) to obtain pTBC-Ha-RAS^Leu61-SEAP.

**Cell Cultures and Transfections—NIH3T3-HER1 cells (17) were grown in agar cultures containing 25 ng/ml EGF (Sigma), and subclones were isolated after 14 days of incubation. Transformation efficiency of each subclone was assessed by estimating anchorage-independent growth in agar cultures. One clone (HER1 KA1) with a stable transformation frequency of 40% colony-forming units was chosen for further growth in agar cultures. One clone (HER1 KA1) with a stable transformation frequency of 40% colony-forming units was chosen for further analysis. For agar cultures, 10^5 cells suspended in 5 ml of 0.25% (w/v) agar/DMEM were plated into 100-mm bacterial dishes on top of a gelified agar/DMEM bottom layer. Both layers contained 10% (v/v) newborn calf serum. Developing colonies were counted after 14 days incubation.

Inducible Ha-RAS expressing cell lines were obtained by co-transfecting pTBC-Ha-RAS^Leu61-SEAP, pUHD15.1 and pTBC1-Hygro into NIH3T3 cells and selecting in 125 μg/ml hygromycin as described previously (25). Isolated clones were tested for anhydrosterecine (Aceros Chimica, Niddereau, Germany)-regulated p21-Ha-RAS expression by Western blotting, using the polyclonal anti-Ha-RAS antibody C20 (Santa Cruz Biotechnology, Santa Cruz, CA), and by reversible tumorigenicity in vitro and in vivo. The cell line N59.4.15, with tightly regulated Ha-Ras expression, was selected for further experiments. HER1-v-src cells were obtained by electroporating the expression plasmid pMSsrc (26) into 3T3-HER1 cells and selecting for transformants using standard focus forming assays (25).

**Panning and Antibody Treatment—**For panning, 100-mm bacterial plates were first treated with 10 ml of a 20% bovine serum albumin and 10 mM EDTA solution in PBS and resuspended in 50 ml of PBS containing 100 μg/ml horseradish peroxidase goat anti-mouse IgG (Dianova, Hamburg, Germany). After 30 min on ice, cells were washed 3 × in PBS and resuspended in 50 ml of 0.1 M Tris/HCl, pH 8.5. Fifty microliters of a solution containing the horseradish peroxidase-specific substrate were added to the cells, and light emission was measured for 1 s in a Micro-Lumat LB960 P lumino

**Amplification of Upstream Sequences—**5'-RACE was performed with 1 μg of total RNA using the 5'-RACE kit from Life Technologies, Inc., following the manufacturer's instructions. The specific CD2 reverse primers were as follows: 5'-CAAGTTTCTCTGGCACC3' and 5'-GGTTCTCAAGGCTCTCATCCTC'-3' (nested). Amplification reactions were allowed to proceed for 20 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and 30 cycles at 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min. The RACE products were cloned into pCR2.1 vectors (Invitrogen, San Diego, CA) and sequenced as described previously (8).

**RESULTS**

**Construction of a Gene Trap Retrovirus Expressing Two Selectable Genes from Dicistronic Transcripts (U3CD2SEAP)—**A U3CD2SEAP gene trap vector was derived from the Moloney murine leukemia virus-based pGgtkNeoU3His(en-) (2) by replacing the His gene with coding sequences for the human cell surface receptor (CD2) and for the secreted human placental alkaline phosphatase (SEAP) (20, 21). To enable translation of both genes from dicistronic transcripts, the SEAP gene was separated from CD2 by an internal ribosomal entry site (IRES) (21). This gene trap allows positive and negative selection as well as a quantitative monitoring of transcriptional activity. To enable selection for high virus titer producer cells, the viral construct also contained an independent selectable marker gene (neo) controlled by an herpes simplex virus thymidine kinase promoter between theLTRs (Fig. 1A) (2).

The plasmid was transfected into GP + E86 helper cells, and supernatants from neomycin-resistant lines producing recombinant virus were used to infect HER1KA1 cells. As has been shown in previous studies, virus replication and long terminal repeat (LTR)-mediated duplication places the sequences inserted into U3 just 30 nucleotides downstream from the flanking cellular DNA. This enables their expression from integrations into transcribed genes (Fig. 1A) (2, 30).

**Selection for and against CD2 Expression Identifies Clones with U3CD2SEAP Proviruses Integrations in EGF-Responsive Genes—**HER1KA1 cells infected with U3CD2SEAP retrovirus were selected for CD2 expression by "panning" using a fusion protein that contained the natural ligand of CD2 (LFA3) and human IgG1 (LFA3-IgG1) (20, 31). The recovered cells, comprising at least 2 × 10^4 transcribed integrations, were exposed to transforming concentrations of EGF (25 ng/ml) (16) for 72 h and then treated with monoclonal anti-CD2-antibody and complement. This was expected to eliminate integrations into constitutively expressed genes and thus enrich for integrations into genes repressed by transformation. The surviving cells were subdivided into 48 pools which were assayed for EGF-responsive integrations by estimating at least two of the following parameters: (i) binding of aminothiolithionibonucleoside bromide-treated SRBC to the CD2 receptor (SRBC rosetting) (28); (ii) CD2-receptor density using a recently described enzyme-linked immunosorbent assay for cell-surface receptors (29); and (iii) measuring SEAP levels in culture supernatants using a chemoluminescent assay (25). Two pools were selected and plated into semi-solid cultures to isolate clones. Twelve clones of each pool were expanded and analyzed by Southern blotting. The clones HER1KA1/6.1, HER1KA1/11, and HER1KA1/3 were unique and had 5, 1, and 1 proviral integrations, respectively (data not shown). Since HER1KA1/6.1 and HER1KA1/11 were particularly sensitive to EGF, they were chosen for fur
To exclude the possibility that more than one provirus had disrupted an expressed gene in the HER1KA1/6.1 clone, mRNA recovered from both EGF-treated and untreated cells was analyzed by Northern blotting. When hybridized to an IRES-specific probe, two major transcripts were detected in absence of EGF, e.g. a 4.3-kb cell-provirus fusion transcript initiating in a nearby cellular promoter and terminating in the polyadenylation site of the 5'-proviral LTR, and a 5.5-kb transcript initiating at the internal thymidine kinase promoter and terminating in the polyadenylation site of the 3'-LTR (Fig. 1A). Although this suggests that CD2 might be translated from both transcripts, previous studies have clearly shown that only the reporter genes of the 5'-LTR are efficiently translated from cell-provirus fusion transcripts (1, 2, 32, 33). In contrast to the proviral transcripts that continued to be expressed in EGF-treated cells, fusion transcripts disappeared almost completely, suggesting that they were repressed by EGF (Fig. 1C). The results indicate that in HER1KA1/6.1 cells only one out of five proviral integrations is expressed from a nearby cellular promoter.

**Proviruses in HER1KA1/6.1 and HER1KA1/11 Cells Disrupt Known Genes**—To ascertain that the gene traps had disrupted expressed cellular genes in HER1KA1/6.1 and HER1KA1/11 cells, the cellular components of the respective cell-provirus fusion transcripts (Fig. 1C) were amplified by 5’-RACE by using CD2-specific primers (8). When the amplification product from HER1KA1/6.1 was hybridized to RNA from wild type HER1KA1 cells, it detected a major cellular transcript of 4.5-kb which was readily repressed by EGF (Fig. 2A, lanes 1 and 2). Similarly, sequences amplified from HER1KA1/11 cells hybridized to a cellular transcript of 5.2 and 5.7 kb (Fig. 2B, lanes 1 and 2).

Sequence analysis of the cloned HER1KA1/6.1 and
HER1KA1/11 amplification products revealed a 100% homology to the first exon of the Timp3 gene (18) and to the first exon of the Col1A2 gene (19). Interestingly, both RACE products exhibited a 103-nucleotide deletion that included the cell DNA-provirus junctions, the translation initiating AUG of CD2, and the signal peptide encoding sequence of CD2. This resulted in an in frame fusion of the cellular sequence to the remaining CD2 sequence, suggesting that the gene trap was activated by mRNA splicing. Indeed, as has been previously observed with other marker genes inserted into U3 regions, the CD2 gene contains a 3'-splice consensus sequence exactly at the site of fusion (Fig. 3). Thus, in both cases the gene trap must have integrated into the first introns of the respective cellular genes (Fig. 3). Moreover, the fusion proteins were still capable of LFA3 and anti-CD2 antibody binding. This suggested that the CD2 moiety was directed to the membrane by the fused signal sequences of the secreted TIMP3 and COL1A2 proteins. Indeed, as shown in Fig. 3, both RACE products contained the signal sequences of the respective proteins (Fig. 3, underlined) (34, 35).

Repression of TIMP3 and COL1A2 Requires EGF—To test whether TIMP3 and COL1A2 repression was linked to EGF or instead was a consequence of cellular transformation, we ana-

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3 H. E. Ruley, personal communication.

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Fig. 3. Gene trap activating mechanism in HER1KA1/6.1 and HER1KA1/11 cells. A (top), predicted gene trap integration site in the first intron of the mouse Timp3 gene (18). In frame fusion of Timp3 and CD2 sequences is achieved by splicing nucleotide 3283 of Timp3 to nucleotide 104 of the provirus which eliminates the CD2-AUG. The 3'-splice consensus sequence of CD2 is shown below the U3 region of the provirus. Black boxes are Timp3 exons, and solid lines are Timp3 introns. Bottom, sequence homology between the 5'-RACE amplification product of HER1KA1/6.1 cells and the first exon of Timp3 (GenBank™ accession number U19462). The exon/intron junction of Timp3 and the site of Timp3/CD2 gene fusion are indicated by arrows. The AUG of the fusion transcript and the 5'-splice consensus sequence of Timp3 are shaded. B (top), predicted gene trap integration site in the first intron of the mouse Col1A2 gene (34). In frame fusion of Col1A2 and CD2 sequences is achieved by splicing nucleotide 1556 of Col1A2 to nucleotide 104 of the provirus which eliminates the CD2-AUG. Black boxes are Col1A2 exons, and solid lines are Col1A2 introns. Bottom, sequence homology between the 5'-RACE amplification product of HER1KA1/11 cells and the first exon of Col1A2 (GenBank™ accession number K01832). The exon/intron junction of Col1A2 and the site of Col1A2/CD2 gene fusion are indicated by arrows. The AUG of the fusion transcript and the 5'-splice consensus sequence of Timp3 are shaded. Signal sequences for both genes are underlined.

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Fig. 4. TIMP3 and COL1A2 expression in transformed cells. A, Northern blot analysis of v-SRC transformed cells. HER1v-SRC cells were obtained by electroporating pMSsrc into HER1 cells as described previously (12) and by selecting for transformed clones. Transformed HER1v-SRC cells were exposed to 25 ng/ml EGF for 48 h. RNAs were hybridized on Northern blots to HER1 KA1/6.1 (TIMP3) RACE product-(top), HER1 KA1/11 (COL1A2 RACE product-(center), and v-SRC (bottom)-specific probes. B, Northern blot analysis of Ha-ras-transformed N59.4/15 cells. N59.4/15 were propagated in 10 ng/ml anhydrotetracycline (Atc) for 5 days to switch off ras expression. RNAs were hybridized on Northern blots to 32P-labeled TIMP3 (top), COL1A2 (center), and β-actin-specific probes.
lyzed their expression in the presence of the EGF receptor kinase-specific antagonist PD153035 (36). As shown in Fig. 2 (lanes 3 and 4), PD153035 prevented Timp3 and Col1A2 transcripts from responding to EGF, indicating that EGFR signaling is required for repression.

To address the possibility of nonspecific repression, we transformed the original EGFR overexpressing HER1 cells with the \textit{v-src} oncogene using the expression plasmid pMSsrc (26). Highly transformed cells were isolated from a focus, exposed to EGF, and analyzed by Northern blotting. Fig. 4A shows that HER1v-src cells continued to express Timp3 and Col1A2. More importantly, both transcripts were readily repressed by EGF, indicating that \textit{v-src}-induced transformation does not modify the response to EGF. We conclude that down-regulation of Timp3 and Col1A2 requires EGFR activation.

Since downstream EGFR signaling involves at least two distinct pathways, one of which includes the protooncogene \textit{c-ras} (37, 38), we wanted to determine whether activated RAS could mimic the EGF-mediated suppression of Timp3 and Col1A2 expression. To this end, the transforming human Ha-RAS\textit{Leu-61} oncogene (39) was transduced in NIH3T3 cells using an expression vector with a tetracycline-repressible promoter (24). A cell line (N59.4/15) expressing a tightly controlled Ha-RAS\textit{Leu-61} was used to analyze Timp3 and Col1A2 transcription. Withdrawal of anhydrotetracycline (Atc) resulted in oncogenic transformation associated with a 20-fold increase in p21Ha-RAS-Leu-61 (data not shown) and a significant reduction of Timp3- and Col1A2 mRNA (Fig. 4B). A similar repression was observed in cell lines conditionally transformed with oncogenic \textit{c-raf1} which is a downstream target of ras (data not shown). These results suggest that EGF engages \textit{c-ras} to suppress Timp3 and Col1A2 expression.

**TIMP3 Antagonizes the Transformed Phenotype of EGF and RAS-transformed Cells**—While overexpression of Col1A2 has recently been shown to repress \textit{ras}-induced transformation in NIH3T3 cells (40), it is still unclear whether TIMP3 has a similar effect in fibroblasts. Since TIMP3 has been implicated in both oncogenic transformation and tumor suppression in systems ranging from chicken embryo fibroblasts to human colon carcinoma cells (18, 41–43), it was important to determine whether TIMP3 would revert a transformed phenotype induced by EGFR signaling. To this end, Timp3 coding sequences were cloned into the retrovirus expression vector pBabe-puro (44) to obtain pBabeTIMP3 (Fig. 5A). pBabeTIMP3 was packaged into virus particles by transient transfection into BOSC23 cells (12) and supernatants thereof were used to infect HER1KA1, HER1KA1/6.1, and tetracycline-inducible Ha-RAS expressing N59.4/15 cells (Fig. 5A). Mixed populations of infected cells were recovered following selection in puromycin and assayed for focus formation and anchorage-independent growth in the presence or absence of EGF or tetracyclin. A 2–3-fold reduction of colony growth was observed in TIMP3 expressing HER1KA1 and N59.4/15 cells in which endogenous Timp3 expression was repressed by EGF and activated ras, respectively. However, this effect was reduced in HER1KA1/6.1 cells both in the presence or absence of EGF (Table I, Fig. 5B). Since these cells exhibited a transformed phenotype even in absence of EGF (Table I, Fig. 5B), it is likely that mutations acquired by other genes antagonize the tumor suppressor function.

![Fig. 5. Exogenous TIMP3 expression in HER1KA1-, HER1KA1/6.1-, and N59.4/15 cells. A (top), structure of pBabeTIMP3 proviruses and predicted transcript. Bottom, Northern blot analysis of proviral TIMP3 transcripts. RNAs were hybridized to 32P-labeled Timp3 and β-actin probes. Even and odd lanes contain RNAs from cells infected with pBabeTIMP3 and pBabe-puro, respectively. Note the almost complete repression of endogenous TIMP3 transcripts (lanes 1, 3, and 5). B, colony formation in soft agar in presence and absence of exogenous TIMP3. HER1KA1-, HER1KA1/6.1-, and N59.4/15 cells were transduced with Timp3 cDNA using the retroviral expression vector Babe-puro (44). Cells obtained after selecting for 7 days in 5 μg/ml puromycin were grown in agar cultures as described under “Materials and Methods.” Colonies were evaluated after incubating for 14 days using dark field microscopy at 40× magnification.](image-url)
10^6 cells were analyzed for focus formation and growth in soft agar as described under "Experimental Procedures." EGF concentrations were 25 ng/ml. Ha-RAS^{Leu-61} was induced in N59 4/15 cells by removing anhydrotetracycline (Atc) from the culture medium. Control cells were obtained by infecting with pBabepuromycin and selecting in puromycin. TIMP3 expressing cells were obtained by infecting with pBabeTIMP3 and selecting in puromycin. Results are means ± S.D. of 2–4 independent experiments.

| Cells | Focus formation | Growth in soft agar | Efficiency of colony formation in soft agar |
|-------|----------------|---------------------|------------------------------------------|
| HER1KA1 | –EGF | 15 ± 7 | 0 | 0 |
|        | +EGF | 205 ± 7 | 40,000 ± 2,000 | 0.94 |
| HER1KA1/6.1 | –EGF | 64 ± 14 | 940 ± 85 | 0.94 |
|        | +EGF | 393 ± 30 | 54,000 ± 2,000 | 38 ± 17 |
| N59 4/15 | –Atc | 0 | 0 | 0 |
|        | –Atc | 900 ± 82 | 59,000 ± 7,000 | 350 ± 147 |

**TABLE I**

Repression of focus formation and colony growth in soft agar by TIMP3

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