Transformation by Ras Suppresses Expression of the Neurotrophic Growth Factor Pleiotrophin*

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An 18-kDa protein (p18) was detected in lysates and conditioned medium from contact-arrested NIH 3T3 fibroblasts, but was not detected when the cells were transformed by the oncogene ras. Analysis of transformation-defective cell clones generated after mutagenesis of the ras-retroviral vector used to transduce the ras gene showed an inverse correlation between p18 expression and the degree of transformation. p18 expression was high in non-transformed clones, intermediate in a partially transformed clone, undetectable in fully transformed cells, but increased as the cells approached confluence and peaked 2–4 days after confluence. Microsequencing of partially purified p18 identified it as the developmentally regulated neurotrophic factor pleiotrophin. In further experiments, pleiotrophin was undetectable or almost undetectable in medium from fully transformed cells expressing the oncogenes v-src, truncated c-ras, activated c-fms, or polyomavirus middle tumor antigen; it was low but easily detectable in medium from SV40 large tumor antigen-expressing cells, which form soft agar colonies but not foci. Thus, pleiotrophin expression in NIH 3T3 cells is associated with quiescence, and suppression of pleiotrophin is related to oncogenic transformation.

The process which causes a proliferating cell to stop dividing is arguably the most important prerequisite for the orderly development of multicellular organisms. During embryogenesis, precursor cells which will form each tissue or organ proliferate and migrate to their predetermined position. They then cease to divide and commence to differentiate into their mature functional form. They also await or actively promote the arrival of other tissues, including blood vessels and nerve fibers, by secreting additional factors. Similar events occur during wound repair as the appropriate cell types proliferate only until they reach the boundaries of the original tissue. In tissue culture, cells which display good growth control and contact-inhibition cease to divide and commence to differentiate into their mature function. This work addresses these questions by examining a factor thought to be involved in both development and tumor formation which appears to be a marker for normal contact-inhibition and quiescence.

Pleiotrophin (1), also known as heparin-binding growth-associated molecule (HB-GAM) (2), heparin-binding growth factor 8 (3), heparin-binding neurotrophic factor (HBNF) (4), and osteoblast-specific protein-1 (OSF-1) (5), has been isolated by several groups based on divergent biological characteristics. The protein is a 136-amino acid neurotrophic cytokine which is capable of stimulating neurite outgrowth in cultured neurons from various sources (10–12). Pleiotrophin is reported to have mitogenic and angiogenic activity for numerous cell types (1, 3, 13–15) and to be produced by human tumors (14, 16, 17), but these results are controversial. It can bind to the transmembrane proteoglycans N-syndecan (18) and syndecan-1 (19), and to the shuttle protein nucleolin (20).

This report describes a new isolation of pleiotrophin based on quite different characteristics. The work began as an investigation of the characteristics of cells transformed by the oncogene ras (for review, see Refs. 21–23). The ras genes encode a family of 21-kDa membrane-associated GTPases. A single point mutation at any of several critical positions inhibits the GTPase function and activates the transforming potential of p21ras (24, 25). Mutations in ras have been implicated in a variety of human tumors (26, 27). In this report, pleiotrophin is identified as a confluence-specific protein secreted by normal cells which is not produced when the cells are transformed by ras or other oncogenes.

EXPERIMENTAL PROCEDURES

Cell Lines—NIH 3T3 cells or derivatives thereof were used in all experiments. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)† (Life Technologies, Inc.) containing 10% donor calf serum and maintained in a 10% CO₂ incubator. ras cells expressed a transforming c-Ha-ras cDNA activated by substitution of leucine at codon 61 (28). The ras gene was expressed from an integrated retroviral vector which also carries a G418 resistance marker (29). Ras N.62 cells were obtained by pooling more than 50 G418-resistant infected colonies; Ras N.62 cells were from a single colony (28). Pleiotrophin was observed in both NIH 3T3 cells and in NIH 3T3 cells infected with the control pDOL retrovirus (29) which carried no insert. To obtain the transformation-defective RM (ras mutant) cell lines in Fig. 1, the retroviral plasmid pRPD (28) containing the rasneo−61 insert was mutagenized with hydroxylamine or shortwave ultraviolet light prior to transfection

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† The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; pyMT, polyomavirus middle tumor antigen; svLT, simian virus 40 large tumor antigen; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
into packaging cells. Viral supernatants were used to infect target cells and G418-resistant cell lines which were morphologically non-transformed or untransformed were isolated for further study (30). v-src transformed cells were made by infection with a recombinant retrovirus produced by the P2 V5 cell line (31), selection with G418 (Life Technologies, Inc.) and pooling of resistant colonies. Cells transformed by c-src1 gene activated by deletion of codons 3–333 were generated in similar with a retrovirus produced by the P2 LN42K cell line (32). Cells transformed by the middle tumor antigen of polyomavirus (pyMT) were made with a retrovirus from the P2 MT12 cell line (33). Cells transformed by c-fms expressed from an SV40-based expression plasmid (34) were obtained from Gary C. Taylor, Mount Sinai Hospital Research Institute, Toronto. Cells transformed by the large tumor antigen of simian virus 40 (svLV) expressed from a retroviral vector (35) were obtained from Myles Brown, Dana-Farber Cancer Institute, Boston, MA.

**Soft Agar Assays**—2 × 10^4 cells were seeded into 2 ml of top agar (DMEM, 10% donor calf serum, 0.3% Difco bacto-agar) and layered onto 5 ml of pre-solidified 0.6% bottom agar in 60-mm dishes.

**Contact Inhibition**—Non-transformed cells were allowed to proliferate until they formed a flat, confluent monolayer and then were layered onto 2–3 days at confluence prior to labeling or analysis. Transformed cells were grown until very dense, such that the number of cells per plate was at least 4-fold higher than the number of non-transformed cells on a confluent plate.

**Metabolic Labeling**—As noted in the figure legends, cells in 35-mm dishes were washed 3 times with methionine-free DMEM (Life Technologies, Inc.) and then were metabolically labeled with [35S]methionine (New England Nuclear) in 600–700 μl of methionine-free DMEM at 250 μCi/ml with or without 10% calf serum for 4 h or 8 h.

**Cell Lysis**—After labeling, the conditioned medium was removed and the cells were washed twice with 2.5 ml of 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2. The cells were then lysed in 500 μl of RIPA-1 buffer (10 mM phosphate buffer, pH 7.2, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 150 mM NaCl, 1 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, 0.3 units/ml aprotinin, and 0.1 mM leupeptin). The lysates were cleared by centrifugation and frozen. 10 μl of each sample was always frozen separately for trichloroacetic acid precipitation analysis. Samples were normalized for equal amounts of [3H]label incorporated into trichloroacetic acid precipitable material. Typically, 4–8 × 10^4 cpm of each lysate were used for further analysis.

**Conditioned Medium**—Conditioned labeling medium was removed after the labeling period and cleared by centrifugation. For immunoprecipitations, each sample was mixed with 600 μl of lysis buffer, frozen on dry ice, and stored at −80 °C. From then on, the samples were treated in the same manner as the cell lysates. For other analyses, the samples were frozen immediately without lysis buffer. 10-μl aliquots were frozen separately for trichloroacetic acid analysis. Samples of conditioned medium were normalized among themselves for equal trichloroacetic acid precipitable counts. Typically, 1–7 × 10^6 cpm were used.

**Anti p21 ras Immunoprecipitation and Analytical Precipitation of p18**—Immunoprecipitations of p21 ras were carried out essentially as described (36) with rat anti-p21 monoclonal antibody YAB6–172 (37) or normal rat serum, and a secondary affinity-purified goat anti-rat IgG antibody (Cappel) pre-coated onto Protein A-Sepharose CL-4B beads (Sigma). The samples were analyzed by electrophoresis on 13 or 15% SDS-polyacrylamide gels.

**Poly(A)-Sepharose 4B Precipitation of p18**—Poly(A)-Sepharose 4B beads (Pharmacia 17-0860-01) were swelled in water, equilibrated overnight at 4 °C in RIPA-2 buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 1 mM sodium fluoride, and 0.02% sodium azide), rinsed three times with RIPA-2 and brought up to a final 35% slurry in RIPA-2. Poly(A)-Sepharose 4B from Sigma had significantly less capacity for binding p18.

In a typical experiment, samples of labeled conditioned medium containing equal trichloroacetic acid precipitable counts were brought to 500 μl with DMEM. An aliquot of the poly(A)-Sepharose 4B bead slurry was added and the total volume was brought to 1 ml with additional RIPA-2. (For quantitative experiments, the volume of bead-seeded medium used to determine precipitable counts is not critical. Generally, a bed volume of 33–120 μl was used.) The incubation also included 0.2 mM phenylmethylsulfonyl fluoride and 0.1 μg/ml aprotinin. The samples were incubated at 4 °C for 3 h on a rocker and then spun briefly at low speed in a microcentrifuge. After removal of the supernatant, the beads were washed three times with RIPA-2 and once with Protein buffer (1 mM Tris-HCl, pH 7.25, 0.005 mM EDTA). GSD electrophoresis loading buffer (6.6% SDS, 33% glycerol, 0.051 g/ml diethiothreitol) was added (1 volume to 2 bead bed volumes). The samples were boiled for 3 min, spun through Spin-X 0.45-μm cellulose acetate filters (Costar) for maximum recovery and separated on a 15% gel.

**Western Blots**—Cells were washed 3 times with DMEM and then incubated in fresh DMEM for 8 h. Conditioned media were collected, cleared by centrifugation, and frozen. Samples of medium were assayed for total protein by the Bio-Rad Protein Assay and normalized to each other. SDS-PAGE was carried out on 15% gels. Transfer onto an Immobilon P membrane (Millipore) and analytical Western blots for pleiotrophin were performed according to standard protocols. Blots were probed with anti-pleiotrophin antibodies 3D1 and mouse anti-human 1B42F11 (Pierce) as the primary antibody and mouse anti-goat IgG conjugated with horseradish peroxidase (Pierce) as the secondary antibody. Detection was by Enhanced Chemiluminescence (ECL) (Amersham).

**Large Scale Preparation of p18**—NIH 3T3 cells were plated into 33 150-mm dishes and allowed to come to confluence over 3 days. At confluence, each plate of cells was fed with DMEM plus 10% calf serum, incubated overnight, and frozen separately in 10 ml of DMEM (without serum) for 8 h. The conditioned medium from all plates was pooled, cleared by centrifugation, frozen in dry ice/ethanol, and stored at −80 °C. An aliquot of the medium was assayed to determine the binding capacity of the poly(A)-Sepharose 4B beads for the p18 in this preparation. The bulk precipitation mixture consisted of 330 ml of conditioned medium, 350 ml of RIPA-2 buffer, 660 μl of 20 mM phenylmethylsulfonyl fluoride, 350 μg aprotinin, and 24 ml of a 50% suspension of poly(A)-Sepharose 4B beads (12–ml bed volume, approximately 3.2 g dry weight), distributed among three 200-ml disposable centrifuge bottles (Falcon). These were rocked at 4 °C for 6 h. They were then spun at 3000 rpm in a bench-top Beckman centrifuge at 4 °C for 15 min, the beads were allowed to settle for 10 min and most of the supernatant was removed. The beads were pooled, washed with RIPA-2 buffer and transferred to six 15-ml Falcon tubes, and washed twice more with RIPA-2. Each tube, now containing about 3 ml of bead suspension, was frozen once with 3 ml of 20 mM Tris-HCl, pH 7.25, 8 M urea, 16% Nonidet P-40, 0.1 mM EDTA, and once with 15 ml of 20 mM Tris-HCl, pH 7.25, 400 mM NaCl, 0.1 mM EDTA. These two washes had previously been shown to strip other proteins from the beads without removing any p18. The beads were then washed twice with Protein buffer, pooled in two 15-ml tubes and washed once more with Protein buffer. The supernatant was aspirated off the top of the beads. Approximately 11.5 ml of total bed volume of beads was recovered. 1.5 ml of GSD was added to each tube and the samples were boiled for 7 min. The samples were pooled, transferred to one Costar 50-ml Spin-X filter tube, and centrifuged to separate the liquid from the beads. Approximately 6.25 ml of liquid sample were recovered.

The SDS-PAGE conditions were adapted from a method of Schagger and Von Jagow (38) to improve separation for pleiotrophin and other proteins. A 15% resolving gel (National Diagnostics, 30%:30% acrylamide: bis) and a long 4% stacking gel were employed. An additional problem encountered with slab gels was that the small proteins typically spread across the slab into wide bands. Consequently, to physically limit diffusion, the gel was cast in a 10-ml disposable polystyrene pipette (Falcon) from which the tip had been broken off. The resolving gel extended from the 9.2-ml mark up to the 5.2-ml mark. The stacking gel extended to the 1.5-ml mark. 5.8 ml of the recovered sample was loaded above the stack. The pipette was supported in a Bio-Rad Protein II xi electrophoresis chamber with the top raised to allow for the extra height of the pipette. Electrophoresis was performed at 30 mA for 16 h. The pipette was then cracked with pliers, and the tubular resolving gel was removed. The gel was laid on a sheet of polyvinylidine difluoride membrane (Biodyne A, and then set up in a slab gel Western sandwich, allowing the proteins to migrate laterally out of the tube. Electrophoresis was performed at 0.25 A in 25 mM Tris, 192 mM glycin, 2% methanol, 0.1% SDS. After transfer, the membrane was stained with 0.1% Amido Black 10B. The correct band was identified by comparison to prestained protein standards run in parallel tubes and was excised for microsequencing.

**Protein Microsequencing**—The preparative sample of p18 on the polyvinylidene difluoride membrane was digested with trypsin in situ. The peptides were separated by high performance liquid chromatography and individual peptides were analyzed on an Applied Biosystems model 477A Protein Sequencer with a model 120A on-line phenylthiohydantoin-amino acid analyzer. Analysis was carried out by William S. Lane, John Nevey, Renee Robinson, and co-workers at the Harvard Microchemistry Lab, Cambridge, MA.
RESULTS

Initial experiments identified a protein with an apparent molecular mass of 18 kDa (p18), which appeared during immunoprecipitation of p21Leu-61 as a prominent band in non-transformed control cells but not in ras-transformed cells. NIH 3T3 cells expressing an activated ras p21Leu-61 were generated by infection with a recombinant retroviral vector and were fully transformed (28). Control DOL retrovirus-infected NIH 3T3 cells were morphologically normal and formed a contact-arrested monolayer. As shown in Fig. 1A, the ras-transformed cells displayed an easily distinguishable ras p21Leu-61 band (lane 6), which ran with a faster mobility than the endogenous p21c-Ha-ras (39) and which was absent in DOL cells (lane 1). In the immunoprecipitates from contact-arrested DOL cells, however, there was a prominent band at 18 kDa (lane 1), which was entirely absent from immunoprecipitates from the ras cells (lane 6). When normal rat serum was substituted for anti-p21 antibody, the 18-kDa protein was brought down in the same manner from DOL but not from ras cells (data not shown). This indicated that p18 was not an alternative form of p21ras, was not bound to p21, and was not an antigenically related protein.

Later experiments, in which the primary and secondary antibodies were omitted, showed that p18 was binding primarily to the Protein A-Sepharose CL-4B beads used to pull down the antibodies (data not shown). Further investigation also proved that p18 was not related to the retroviral vector, since the pattern of p18 expression was identical in uninfected NIH 3T3 cells and in DOL-infected cells (Fig. 2). This intriguing apparent correlation between ras transformation and the disappearance of p18 called for further study.

p18 Expression in Ras Mutant Cell Populations—A mutagenesis strategy, which was designed to generate transformation-defective mutants of the rasLeu-61 gene by mutagenizing the ras retroviral plasmid (30), produced a variety of infected cell clones covering a range of phenotypes, including fully transformed, partially transformed, non-transformed, and cold-sensitive for transformation. These clones were examined to define the relationship between p18 expression and ras transformation. The results are summarized in Table I. As shown in the immunoprecipitations in Fig. 1A, cells which produced no stable ras p21Leu-61 from the mutagenized integrated retrovirus, and which were morphologically normal (lanes 3 and 4), had a p18 band identical to that in control DOL cells (lane 1), whereas cells which were transformed and which were still synthesizing p21Leu-61, did not have a p18 band (lanes 2 and 5). Cell clone RM.37 was cold-sensitive for transformation: the

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**FIG. 1.** p18 is expressed in contact-inhibited fibroblasts but not in ras-transformed fibroblasts. Cells were labeled with [35S]methionine for 4 h in methionine-free medium without serum prior to lysis and immunoprecipitation with anti-p21Leu-61 antibody. 32 and 37 refer to the temperatures at which the cells were cultured and labeled. The characteristics of each clone are summarized in Table I. A, expression of p18 in cells carrying mutagenized ras-retroviruses which are defective for transformation. Lane 1, control DOL infected cells; lane 2, transformed clone RM.26; lane 3, contact-inhibited RM.27; lane 4, contact-inhibited RM.28; lane 5, transformed RM.33; lane 6, non-mutagenized, transformed N.62 cells expressing p21Leu-61. B, expression of p18 in a cell line which is cold-sensitive for transformation. Lanes 7, contact-inhibited RM.35; lanes 8, cold-sensitive clone RM.37, which is transformed at 37 °C but morphologically normal and contact-inhibited at 32 °C; lanes 9, transformed RM.34. C, relationship between p18 expression and maximum cell density. Lane 10, contact-inhibited RM.40; lane 11, transformed RM.42; lanes 12 partially-transformed RM.44; lane 13, contact-inhibited RM.45.

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**Table I**

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<th>Cell clone</th>
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<th>Soft agar c</th>
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a Phenotype: F = flat, morphologically normal; Tx = transformed; PT = partially transformed (the cells are rounded and grow to high density but do not overgrow the monolayer).
b Overgrow: the cells overgrow the monolayer and pile up, and/or form foci on a non-transformed monolayer.
c Soft agar: ability of cells to form colonies in 0.3% agar; nt = not tested.
d p18/PTN: presence or absence as shown in Figs. 1 and 6; +/- = intermediate level of p18.
cells were morphologically transformed, overgrew the monolayer, and formed colonies in soft agar at 37 or 39 °C, but were morphologically normal, contact-inhibited, and incapable of growth in soft agar at 32 °C (data not shown). Fig. 1B demonstrates that at 37 °C these cells made a significant amount of \( \text{ras} \) p21Leu-61 but very little p18; in contrast, at 32 °C the level of stable p21Leu-61 was low but the amount of p18 was significantly increased (lanes 8). In cells which were morphologically normal at both temperatures, the level of p18 was high at both temperatures (lanes 7). In cells which were transformed at both temperatures, the level of \( \text{ras} \) p21Leu-61 was the same at both temperatures and there was no sudden appearance of p18 at 32 °C (lanes 9). Finally, as shown in Fig. 1C, there appeared to be a relationship between the level of p18 production and contact- or density-inhibition of growth. The cell clone RM.44 (lanes 12) produced p21Leu-61 at a level equivalent to that of the fully-transformed cells (lane 11). However, the phenotype of RM.44 was “partially transformed.” The cells grew to a high density but were incapable of piling on top of one another in typical \( \text{ras} \)-transformed fashion. They also formed somewhat smaller colonies in soft agar (data not shown). The p18 level in RM.44 cells was likewise intermediate between the level in fully transformed cells (lane 11) and the level in two cell clones which formed normal growth-arrested monolayers (lanes 10 and 13). Thus, among all the cell clones tested, there was a strict inverse correlation between p18 expression and the degree of transformation, as summarized in Table I.

**p18 Is Secreted by Non-transformed Cells**—To determine whether p18 might simply be degraded more rapidly in \( \text{ras} \) cells than in non-transformed cells, the cells were pulse-labeled with \(^{35}\text{S}\)methionine for 1 h, and the labeled was then chased in the presence of cycloheximide to inhibit any further protein synthesis. In lysates from \( \text{ras} \) cells, p18 was not detectable at any time point (data not shown), indicating that p18 was not being produced at all. In lysates from DOL cells, p18 was detectable after the 1-h label, but the labeled band disappeared after a 1-h chase (data not shown). Further investigation revealed that this rapid disappearance of p18 from the DOL lysates was due to secretion of the protein into the medium, as illustrated in Fig. 2. The conditioned media from labeled cells were put through the same immunoprecipitation procedure as the cell lysates, and the results were exactly parallel. p18 was present in the conditioned medium of non-\( \text{ras} \) NIH 3T3 or DOL-infected cells (lanes N and D), but not in the conditioned medium from \( \text{ras} \)-transformed cells (lanes R). In fact, p18 was the major low molecular weight protein in the medium which bound to the immunoprecipitation complex. As expected, the p21ras band was present in the cell lysates but not in the medium from the \( \text{ras} \) cells (lanes R), since p21 is an intracellular protein.

**p18 Expression after Addition of Serum**—Since the normal cells always formed a quiescent monolayer, it was of interest to know if the production of p18 would vary according to whether or not fresh 10% calf serum was added at the beginning of the labeling period. As shown in Fig. 2 (lanes N and D), the p18 band in both the cell lysates and the conditioned medium was more intense in the presence of serum than in the absence of serum in both the cell lysates and the conditioned labeling medium. The six cell lysate samples were normalized to each other to contain the same amount of trichloroacetic acid-precipitable labeled material, as were the six samples of conditioned medium. Therefore, the data suggest that the addition of serum resulted in an increased level of p18 as a percentage of total protein synthesized and secreted during the labeling period.

**p18 Binds to Poly(A)-Sepharose 4B**—In the initial experiments, p18 was found in the anti-p21 immunoprecipitation complex, bound primarily to the Protein A-Sepharose CL-4B beads. Since sequence analysis of p18 would require a large scale isolation, further experiments attempted to maximize the binding capacity for p18. The beads were prepared under various conditions and compared with Sepharose CL-4B alone, with \( \text{Staphylococcus A} \) cells in suspension, and with preparations of other Sepharose reagents (data not shown). In the end, the highest binding capacity for p18 was unexpectedly displayed by the polynucleotide-containing affinity matrix poly(A)-Sepharose 4B. This reagent was therefore used in subsequent experiments.

**p18 Expression and Degree of Confluence**—The next step was to determine whether p18 was always either present (normal cells) or absent (\( \text{ras} \)-transformed cells), or whether the level of p18 might be modulated in normal cells depending on their state of proliferation or confluence. Fig. 3 demonstrates a direct correlation between the proliferation/density state of normal NIH 3T3 cells and the level of p18. The experiment measured the amount of p18 as a percentage of total protein synthesized and secreted during the labeling period. p18 was almost undetectable when the cells were sparse and rapidly proliferating (30% confluence). The amount of p18 increased to an intermediate level as the cells reached 80% and then 100% confluence. The highest level of p18 was found when the cells had already been confluent and growth-arrested for 2–4 days (Fig. 3). Parallel results were observed in cell lysates (data not shown). Both the level of labeled p18 in the normalized samples as determined by autoradiography (Fig. 3) and the absolute level of p18 as determined by scanning of the same gel (data not shown) increased in the same manner.

**Isolation of p18 and Identification as Pleiotrophin**—Poly(A)-Sepharose 4B appeared to be the affinity matrix of choice to isolate p18 for sequence analysis. However, various elution methods failed to remove p18 from the matrix quantitatively (data not shown). While p18 could be eluted by boiling in electrophoresis loading buffer, a large scale preparation yielded 5 ml of sample. When this sample was directly separated by...
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SDS-PAGE under traditional Laemmli conditions (40), p18 was not resolved (data not shown). This probably occurred because proteins below 20 kDa may not be separated from the bulk of SDS in the stacking gel, thereby preventing proper resolution in the separating gel (38). Attempts to remove the 2.2% SDS or to concentrate the sample while preserving p18 were likewise unsuccessful. Eventually, a modified Tricine gel (38), incorporating an extra-long stack and cast as a tube to physically limit lateral diffusion, was employed to resolve p18 successfully from a 5.8-ml sample. p18 was then isolated by direct electrotransfer from the resolving tube gel to a polyvinylidene difluoride membrane. Amino acid sequencing was carried out by William S. Lane at the Harvard Microsequencing facility. Three peptides yielded the sequences shown in Fig. 4. Comparison with the SwissProt and GenBank protein data bases conclusively identified p18 as pleiotrophin. Western blotting with an anti-pleiotrophin antibody. Results with v-fms occurred after the immortalizing event which originally transformed NIH 3T3 cells only in the expression of a single oncogene. Expression was also undetectable or very low when the cells were transformed by a variety of other oncogenes. Likewise, p18 was barely detectable when normal cells were actively dividing. It appeared in quantity only when the cells approached confluence and reached peak expression several days later. Among all the transformed and transformation-defective cell lines tested, there was a direct correlation between the ability of the cells to form a flat, quiescent monolayer and their ability to secrete pleiotrophin.

The identification of p18 as pleiotrophin suggests that p18 was a factor related to contact-inhibition or quiescence. p18 appeared in lysates and conditioned medium from normal NIH 3T3 fibroblasts but was never seen when the same cells were transformed by an activated ras oncogene. Expression was also undetectable or very low when the cells were transformed by a variety of other oncogenes. Likewise, p18 was barely detectable when normal cells were actively dividing. It appeared in quantity only when the cells approached confluence and reached peak expression several days later. Among all the transformed and transformation-defective cell lines tested, there was a direct correlation between the ability of the cells to form a flat, quiescent monolayer and their ability to secrete pleiotrophin.

Because a tumor is the end result of a series of mutations, it is difficult to determine the point at which any particular change occurred. The experiments described here have the advantage that each transformed cell population differs from the parent NIH 3T3 cells only in the expression of a single oncogene. The suppression of the ability to produce pleiotrophin occurred after the immortalizing event which originally produced the NIH 3T3 cell line, and coincident with the transition to full oncogenic transformation. This was absolutely
consistent among the various *ras* clones in Fig. 1, including the cold-sensitive RM.37 where pleiotrophin was present at the non-permissive temperature and absent at the permissive temperature for transformation. It was also consistent among the different oncogenes (Fig. 6), which collectively represent most of the steps of the extranuclear signal transduction pathways which lead to proliferation. The correlation is further substantiated by the observation that the two transformed lines which consistently secreted pleiotrophin at a higher level relative to the other transformed lines did not manifest all the accepted characteristics of full oncogenic transformation. The cells expressing svLT, which represents a different class of oncogenes acting in the nucleus, and the *ras* clone RM.44 could both form colonies in soft agar, but they were incapable of overgrowing a monolayer.

This study suggests that pleiotrophin expression is associated with normal contact-inhibition of growth. It is difficult to rationalize that finding with the previous work which showed that pleiotrophin transforms NIH 3T3 cells. One critical difference is that in the current experiments the expression of pleiotrophin is driven from its native promoter and is subject to normal regulatory control. In the experiments of Fang et al. (14) and Chauhan et al. (50), pleiotrophin was constitutively expressed from the strong SV40 early promoter and cytomegalovirus promoter, respectively. It may be that normal pleiotrophin expression related to contact-inhibited growth occurs only under specific conditions, or only in the G0 phase of the cell cycle. The mitogenic or transforming effect seen by the other investigators may be caused by the experimental circumstances which result in inappropriate timing or conditions of expression, or expression levels which are too high. It has been shown, for example, that interferon-γ, which is ordinarily a growth inhibitor, can stimulate cell growth if no growth factors are present (55).

The relationship between pleiotrophin expression and normal growth control suggests two possibilities. The first is that pleiotrophin is a mediator of growth control, *i.e.* a negative growth factor. As proliferating cells physically come in contact, they would secrete pleiotrophin as an autocrine or paracrine signal to stop proliferation. Failure to express this factor would allow the oncogenically transformed cells to continue to proliferate without regard to cell-cell contact. A very recent report lends some support to this hypothesis by showing that pleiotrophin can inhibit the proliferation of mesenchymal and epithelial cells in cultured rat limb buds (56). The increase in pleiotrophin expression by confluent cells in response to serum (this paper) or to platelet-derived growth factor (57) might then be a signal to reinforce the quiescence by blocking the response of the cells to an increased concentration of mitogens. Several other negative regulators of growth have been described, including tumor growth factor-β and the interferons, and at least part of their function is to abrogate the proliferative stimulus of positive growth factors (see Refs. 58 and 59, for review). Furthermore, post-confluent vascular smooth muscle cells secrete a highly anti-proliferative heparan sulfate species, apparently modulating their own growth in an autocrine fashion (60). Nonetheless, it is clear that pleiotrophin cannot by itself re-stimulate contact inhibition to transformed cells. That is proven by every focus assay in which the confluent monolayer cells secrete pleiotrophin and the transformed cells still grow out as foci. The model implies that both the synthesis and the response to pleiotrophin must be co-repressed in the transformed cells. Such an effect is not unprecedented: the ability to produce and the ability to respond to type I interferons seem to be co-repressed during the proliferative phase of early embryonic development (61).

If pleiotrophin plays a role in contact inhibition, it probably does so in conjunction with other components of the extracellular matrix. Pleiotrophin binds both to heparin and the heparan sulfate-containing proteoglycans, the syndecans (18, 19). The syndecans are involved in cellular association with the extracellular matrix and in presentation of growth factors at the cell surface (62). Heparin also acts at the extracellular matrix (63) and clearly inhibits proliferation when added to a variety of cultured cells, including fibroblasts, smooth muscle cells, and rat cervical epithelial cells (64). It is therefore intriguing to note that this inhibitory effect of heparin on rat cervical epithelial cells is blocked by transformation with the same *ras* retroviral vectors which suppress pleiotrophin expression in the NIH 3T3 cells. Further investigation may show whether pleiotrophin is involved in this inhibitory effect of heparin.

The second possibility is that pleiotrophin may itself be induced by growth arrest in fibroblasts. It could be associated with a differentiation event which occurs after confluence and quiescence. For example, fibroblasts migrating to a wound would proliferate until the wound is filled, cease dividing, and then begin to secrete factors such as pleiotrophin, which would recruit new blood vessels and neurites to the site. If pleiotrophin is viewed as a mitogenic molecule secreted by fibroblasts to influence other cells in paracrine fashion, then its synthesis may be quiescence-specific to prevent it from stimulating the secreting cells. That is, only fibroblasts which are safely lodged in the G0 stage outside the cell cycle would secrete pleiotrophin. In this scenario, the oncogenically-transformed cells would not express it because they never achieve quiescence. Several growth arrest-specific (gas) genes have previously been identified in fibroblasts (58) and at least two of these genes could not be induced if the cells were transformed by the oncogenes *v-src* and *v-fos* (65). This scenario could also explain how inappropriate timing of pleiotrophin expression from a constitutive exogenous promoter (14, 50) could cause fibroblasts to become tumorigenic themselves. In either of the aforementioned scenarios, the results described here are consistent with the tumor data in neuroblastoma (51) and small cell lung carcinoma (52) in suggesting that pleiotrophin expression is a marker for a more growth-controlled, more differentiated, less tumorigenic phenotype.

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