Expression of the c-Harvey ras Oncogene Alters Peptide Synthesis in the Neurosecretory Cell Line AtT20*

(Received for publication, January 6, 1992)

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Ras proteins are enriched in neurosecretory cells suggesting that ras may play an important role in regulating the differentiated properties of such cells. We introduced the human H-ras oncogene, EJ-ras, into the model secretory cell line AtT20 to determine the effects of ras oncogene expression on neuropeptide synthesis and release. We report here that both of these processes are changed in ras-transfected AtT20 cells. Stimulated release of the pituitary hormone corticotropin is reduced, and transcription of the gene encoding its precursor, proopiomelanocortin, is down-regulated. At the same time, expression of other genes, both housekeeping and neural-specific, remain relatively unchanged. The alteration of proopiomelanocortin expression in AtT20 cells following ras oncogene transformation supports the hypothesis that ras may play a role in the determination of the differentiated phenotype of neurosecretory cells.

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§ The abbreviations used are: ACTH, corticotropin; MOPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; POMC, proopiomelanocortin; 8-Br-cAMP, cyclic 8-bromo-AMP.

The protooncogene ras encodes a 21-kDa protein (p21ras) that is highly enriched in terminally differentiated neurons, neurosecretory cells, and muscle (Swanson et al., 1986; Furth et al., 1987; Mizoguchi et al., 1989; Chera et al., 1987). ras has been implicated as a component of regulatory pathways controlling cell division and differentiation, including differentiation to a neuronal phenotype (Bar-Sagi and Feramisco, 1985; Noda et al., 1985; Hagag et al., 1986; Berbcic, 1987; Hanley and Jackson, 1987). Its high expression in mature neurons suggests additional regulatory roles in processes characteristic of these cells, such as electrical excitability and secretion. To address whether p21ras is capable of regulating transmitter release from neurosecretory cells, we have transfected the human H-ras oncogene, EJ-ras (Shih and Weinberg, 1982), into a model neuropeptide synthesizing and secreting cell line, AtT20. These cells are an excitable mouse anterior pituitary cell line that is highly enriched in terminally differentiated neurons, and is a model system for the study of a number of processes underlying neuropeptide physiology. These include hormonal regulation of ACTH and β-endorphin biosynthesis and release (Herbert et al., 1978), posttranslational processing of polypeptide hormone precursors (Roberts et al., 1978), and pathways of secretion (Burgess and Kelly, 1987). We report here that ras oncogene expression results in marked changes in AtT20 cells both at the level of neuropeptide synthesis and secretion.

EXPERIMENTAL PROCEDURES

Culture Conditions and Transfections—All cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C in 5% CO2 and 95% air, except where noted. Transfections were performed by the method of CaPO4 coprecipitation as described (Gorman et al., 1983). Plasmid Edl6.6 was a gift from Ciaho Shih (University of Pennsylvania). Plasmid pRSVNeo was a gift from Bruce Howard (National Cancer Institute).

Preparation of RNA and Northern Blots—RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction, essentially as described (Chomczynski and Sacchi, 1987). Five μg of total RNA was run on 0.66 M formaldehyde, 1% agarose gel in 1 X MOPS buffer (Davis et al., 1986) with recirculation. Gels were transferred onto GeneScreen Plus membranes (Du Pont-New England Nuclear) in 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate). Membranes were then baked for 2 h at 80°C and prehybridized in Hyb-N (Davis et al., 1986) containing 1% SDS and 200 μg/ml denatured salmon sperm DNA at 44°C for >1 h. Hybridization was carried out at 44°C for >16 h in Hyb-N, 1% SDS, 100 μg/ml denatured salmon sperm DNA, and 0.5 to 2 X 106 cpm/ml of labeled probe. The highest stringency washes were at 0.1 X SSC, 1% SDS at 44°C. Autoradiography was performed using Kodak X-Omat AR film to visualize the hybridized probe. The human γ-actin cDNA probe was a gift from Y.-C. Cheng (Yale University). The rat cyclophilin cDNA was a gift from R. Handschumacher. The mouse proopiomelanocortin cDNA clone pMKSU16 (Uhrer and Herbert, 1985) was a gift from M. Uhler. Each cDNA fragment was gel-purified and labeled by random primer extension with DNA polymerase I large fragment and [α-32P]-CTP.

Primer Extension—The primer used in these experiments was a human Harvey ras antisense 20-mer corresponding to positions 137-156 of human H-ras exon III. The oligonucleotide was end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]-ATP and annealed with [α-32P]-ATP at 70°C with an intensifying screen to visualize the products. The assay conditions were as follows: 5 μg of total RNA, 20 ng of labeled oligomer, 1 mM dNTPs, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, 20 mM dithiothreitol, 40 μg/ml actinomycin D, and 4000 units/ml avian myeloblastosis virus reverse transcriptase were added to tubes in a volume of 50 μl. RNAs were heated in the presence of the primer at 95°C for 5 min and quickly chilled on ice. The other reaction components were then added, and reactions were carried out for 2 h at 44°C. After terminating the reactions with 5 μl of 0.25 M EDTA, samples were extracted with phenol/chloroform and precipitated with ethanol. Pellets were dried, resuspended in formamide loading buffer, boiled 5 min, and immediately chilled on ice. Reverse transcripts were then fractionated by size on an 8% acrylamide, 8 M urea sequencing gel in 1 X TBE (90 mM Tris-HCl, 90 mM NaB04, 2 mM EDTA, pH 8.3). pUC18 plasmid digested with MspI and end-labeled with [γ-32P]-ATP served as a molecular weight marker. The gel was dried and exposed to X-Omat AR film at -70°C with an intensifying screen to visualize the products.

Allele-specific PCR—cDNAs were synthesized from total RNA from normal and ras oncogene-transfected AtT20 cells and were

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subjected to PCR amplification with a sense strand primer, specific either for the wild type H-ras sequence or for the mutation found in the human c-Harvey ras oncogene clone EJ6.6 (Stork et al., 1991). A sense strand 13-mer matching the sequence of the human Harvey ras oncogene EJ6.6 (positions 1866–1899) was incubated with a conserved antisense strand (obtained from Pharmacia, see "Primer Extension" above). The allele-specific sense strand probe differs only in the terminal 3'-nucleotide from the wild type gene, a thymidine residue substituting for a guanine residue in the normal H-ras gene. cDNA was subjected to 30 cycles of a temperature program including 1 min at 94°C, 2 min at 58°C, and 1 min at 72°C for the extension reaction in the presence of 2 units of Taq DNA polymerase (Perkin-Elmer Cetus), four dNTPs at 0.25 mM, and 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin, in 100 μl. Fifty μl of each reaction mixture was analyzed by gel electrophoresis in 1.2% agarose in a Tris acetate buffer. The PCR products were then visualized by ethidium bromide fluorescence.

Preparation of Cell Protein Extracts and Western Blots—Extracts containing synapsin I were prepared from wild type or ras-transfected cells as described (Shapiro et al., 1988). Briefly, cells were lysed in hypotonic buffer using a Dounce homogenizer, osmolality was restored, and the homogenate was spun at 12,000 × g for 30 s at 4°C. The pellet was resuspended and rocked for 30 min at 4°C, followed by centrifugation at 150,000 × g for 1.5 h at 4°C. Protein was precipitated by addition of ammonium sulfate to 0.33 g/ml and recovered by centrifugation at 85,000 × g for 20 min at 4°C. After resuspension of the precipitate and dialysis, the Bradford protein assay (Bio-Rad) was used to determine protein concentration. To prepare cell lysates containing p21, cells were harvested and lysed by Dounce homogenization in 50 mM Tris-HCl, 1% Triton X-100 (pH 7.4). Lysates were centrifuged 12,000 × g for 15 min at 4°C. The supernatant was collected and stored overnight at −20°C, thawed, and resuspended at 500 μg/ml in 1% bovine serum albumin/TN. TN buffer is 20 mM Tris, 500 mM NaCl (pH 7.5). After blocking, membranes were incubated with primary antibody for 2 h at 20°C, with shaking in 1% bovine serum albumin/TN. Membranes were then washed twice for 10 min in TN/0.05% Triton X-100, and twice for 10 min in TN. 

**RESULTS**

Establishment of AtT20 Cell Lines Transformed with the c-Harvey ras Oncogene Clone EJ6.6—Plasmid DNA from the ras oncogene clone EJ6.6 (Shih and Weinberg, 1982), derived from a human bladder carcinoma cell line, was cotransfected into AtT20 cells with the selective marker pRSVNeo by calcium phosphate coprecipitation. Cells were selected for their resistance to the antibiotic G418. Unlike sham-transfected controls or cells cotransfected with pRSVNeo and other plasmids, many EJ6.6-transfected cells were observed to survive more than 2 weeks of selection in G418 without undergoing cell division. Their morphologies changed to larger cell bodies with extended and branched processes (Hemmick et al., 1992). Three stable G418-resistant colonies with similar morphology to the surviving nondividing cells were cloned. The frequency of stable transformants of the ras oncogene clone EJ6.6 with pRSVNeo was much lower than that of transfections of the same cells by other genes or by the nontransforming ras gene. This suggests that transfection of the EJ-ras gene results in cells that are unable to continue dividing and may cause terminal differentiation. The frequencies of G418-resistant colony formation in a representative transfection experiment are reported in Table I.

**Table I**

| DNA in precipitate | Number of colonies/10-cm plate |
|--------------------|-------------------------------|
| pRSVNeo (20 μg)    | 200                           |
| pOMC-CAT (20 μg)   | 50                            |
| pRSVNeo (4 μg)     | 45                            |
| pRSVNeo (20 μg)    |                               |
| pRSVNeo (4 μg)     | 60                            |
| c-H-ras protooncogene (20 μg) | 0-1          |
| EJ6.6 (20 μg)      | 0-1                           |
| pRSVNeo (4 μg)     |                               |

AtT20 cells were plated at 10⁶ cells/plate. Cells were transfected the following day according to Gorman et al. (1983). Plates were treated with 600 μg/ml G418 (GIBCO, 43% pure) for 14 days. The anti-H-ras monoclonal antibody Ras 10 (Carney et al., 1988) was used as a probe to detect the presence of p21 protein, followed by 125I-labeled protein A to visualize the bands. As a positive control on the Western blot, we used protein extracted from the human bladder carcinoma cell line T24. The results of this analysis (Fig. 1B) are consistent with an increase in the amount of p21 protein present in the ras-transfected AtT20 cell line.

To determine whether the oncogenic EJ-ras allele is uniquely expressed in the transfected AtT20 clone, we applied a variation of the PCR sensitive to point mutations in the middle base of H-ras codon 12 (Stork et al., 1991). Under the PCR conditions we used, amplification is sensitive to single base mismatches at the 3'-nucleotide of the sense strand 14-mer used in the PCR. The two sense strand oligonucleotides were synthesized that differ only at their 3'-nucleotide corresponding to the middle base of codon 12 of the human H-ras gene.
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were resolved by running on an 8% acrylamide, 8 M urea gel and visualized by autoradiography. Were loaded. Primary antibody was the mouse monoclonal anti-pel line was primed by the 32P-labeled ras Western blots. ras-transfected AtT20 cells express higher levels of p21 than untransfected wild type AtT20 cells. Equal amounts of protein from wild type and ras-transfected AtT20 cells were resolved by running on an 8% acrylamide, 8 M urea gel and visualized by autoradiography. Primary antibody was the mouse monoclonal anti-p21 antibody Ras 10 (Du Pont), which reacts with all three families of oncogene-transformed AtT20 cells. PCRs were performed using a common downstream primer derived from the antisense strand of human ras gene and one of two allele-specific primers derived from the sense strand of the human ras gene (positions 1686–1698) terminating with a guanidine residue (protooncogene specific) or a thymidine residue (oncogene specific). (lanes 3 and 5), pUC19, cut with DdeI, is shown in lane 1 as a marker. Note that only the PCR product in lane 5 (ras-transfected cells, oncogene-specific primer) shows a band on a 1% agarose gel corresponding to a DNA fragment having the predicted size. bp, base pairs.

Each primer was used in a PCR with a common antisense 20-mer 240 nucleotides downstream (see “Experimental Procedures”). Expression of the mutant H-ras allele was identified in cDNA from the EJ-ras transfected R1 cells by the efficient amplification of the predicted 240-base pair fragment by PCR in the presence of the mutant allele-specific primer. Under these conditions, no detectable amplified product was observed with the mutant allele-specific primer when cDNA from wild type human or normal AtT20 RNA was used as template (Fig. 1C). The wild type H-ras sense primer failed to generate a specific PCR product after 30 cycles in either nontransfected or ras-transfected AtT20 cells. However, with additional cycles, a band of the predicted size was observed, indicating the presence of wild type H-ras in both cell types at lower levels (data not shown). We therefore conclude that only the EJ-transfected cell line expresses the oncogenic H-ras allele.

Down-Regulation of ACTH Release and POMC Gene Expression in ras-transfected AtT20 Cell Lines—AtT20 cells synthesize and release the polypeptide hormone ACTH (Herbert et al., 1978). Exposure of AtT20 cells to a number of agents, including corticotropin-releasing factor and cyclic AMP analogues, induces rapid secretion of ACTH from AtT20 cells (Phillips and Tashjian, 1982). In order to determine whether ras oncogene-transformed AtT20 cells are capable of releasing ACTH in response to a known stimulus, normal AtT20 cells and ras-transfected clone R1 were treated with 8-Br-cAMP (1 mM) for 4 h. Aliquots of media were collected and assayed for their ACTH content by radioimmunounassay. As displayed in Fig. 2, panel A, normal AtT20 cells release a readily detectable level of ACTH in response to 8-Br-cAMP treatment. No detectable ACTH was released from the ras-transformed cells under identical stimulating conditions. The loss of the ability of AtT20 cells to undergo stimulated release of ACTH, a characteristic feature of normal AtT20 cells, could be explained by several mechanisms. We speculated
that the loss of ACTH release was due at least in part to a decreased synthesis of the ACTH precursor POMC. To address this possibility we measured mRNA levels encoding POMC in normal and ras-transformed cells. Total RNA from normal AtT20 cells and three ras-transfected clones were assayed by Northern blot analysis with a 32P-labeled mouse POMC cDNA probe. The results (Fig. 2, panel B) indicate that the level of steady state POMC mRNA in two of the three ras-transformed clones has been reduced below the limits of detectability in the assay, estimated to be 100-fold less than that in normal ras cells. When this experiment was repeated using poly(A) RNA from clone R1, no POMC mRNA signal was detectable, even with extended x-ray film exposure times, which allowed the lower abundance high molecular weight POMC mRNA precursors to be visualized in the lane corresponding to wild type AtT20 cells (Fig. 2, panel C). These results are consistent with the loss of stimulated ACTH release and the down-regulation of POMC mRNA occurring at the level of transcription of the POMC gene.

Comparison of Expression of Other Genes in Normal and ras-transformed AtT20 Cells—To determine whether the large change in expression of the POMC gene represents a generalized alteration of ras oncogene transformation, we monitored the expression of several other endogenous genes. In particular, we assayed for the expression of two genes that are ubiquitously expressed in many cell types, the genes encoding γ-actin and cyclophilin. Using the human cDNA clone for γ-actin and the cDNA for cyclophilin as hybridization probes, we found that the levels of γ-actin and cyclophilin mRNA levels in normal and ras-transformed AtT20 cells differ only slightly (Fig. 3A and B). The level of γ-actin mRNA (Fig. 3A) appears to be increased 2–3-fold in ras-transformed cells over wild type AtT20 cells. The level of cyclophilin mRNA remains essentially unchanged (Fig. 3B).

We also measured levels of a differentiated gene product thought to be expressed only in neuronal and neurosecretory cell types, synapsin I. Protein extracts from wild type AtT20 cells and clone R1 were assayed by Western blot with a monoclonal antibody specific to synapsin I. As with γ-actin and cyclophilin, there is only a small difference in expression between the two cell types, with synapsin I appearing slightly decreased in the EJ-ras-transformed cells (Fig. 3C). We conclude from these data that the p21ras oncoprotein specifically affects expression of the POMC gene in AtT20 cells transformed with the EJ-ras gene.

**DISCUSSION**

ras has been shown previously to mediate differentiation in one neuronal cell line, PC-12 (Noda et al., 1985). We investigated the effects of expression of the H-ras oncogene on the phenotype of the pituitary mouse cell line AtT20. We cotransfected pEJ6.6, the EJ-ras gene, into AtT20 cells with the selectable marker pRSVNeo. A small number of G418-resistant clones were obtained. Compared with the frequency of stable transfection when the normal H-ras gene or other plasmids are used, a reduced frequency of appearance of G418-resistant clones with the EJ-ras oncogene was observed (Table I). This is consistent with expression of EJ-ras in transfected AtT20 cells causing terminal differentiation. In the three G418-resistant clones that were established, a number of distinguishing features were observed. The ras-transformed cells and nondividing transfected cells that survived in 600 μg/ml G418 for 2 weeks could be distinguished morphologically from nontransfected cells by their larger size and extensive process growth (Hemmick et al., 1992). A number of retrovirus-infected AtT20 clones expressing the v-H-ras gene also displayed this altered morphology.2

We show that the ras gene is overexpressed in transfected AtT20 cells at the mRNA level and at the level of p21ras protein (Fig. 1). With the application of the technique of allele-specific PCR, we provide evidence that only the ras-transfected AtT20 cells express the point mutation in codon 12 found in the EJ-ras gene (Fig. 1C).

Stimulated release of ACTH in response to 8-Br-cAMP, a hallmark of normal of AtT20 cells, was found to be missing in a ras-transfected morphologically transformed clone (Fig. 2A, for photomicrographs see Hemmick et al. (1992)). We hypothesized that expression of the gene encoding the ACTH precursor proopiomelanocortin was down-regulated. Results from Northern blot analysis support this hypothesis (Fig. 2, B and C). A Northern blot of poly(A)+ RNA from wild type AtT20 and clone R1 cells was hybridized to POMC cDNA and exposed to x-ray film long enough to visualize the lower abundance POMC mRNA precursor (Fig. 2C). Under these conditions no hybridization signal was detected in the ras-transfected clone. This suggests the block to POMC expression is at the level of transcription. A profound change in POMC mRNA half-life from 12–24 h (Birnberg et al., 1983) to minutes could give rise to significantly reduced steady state mRNA levels. However, the absence of any detectable POMC pre-mRNA species is more consistent with a block at the level of transcription. The virtual extinction of POMC mRNA levels in two of three ras-transformed clones analyzed was never observed in normal AtT20 cells or in clones transfected with genes other than EJ-ras. Three control genes, one neural-specific, synapsin I, and two housekeeping, γ-actin and cyclo-

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2 N. C. Birnberg, P. J. S. Stork, and L. M. Hemmick, unpublished results.
phillin, assayed at the RNA or protein level, were found to be largely unchanged (Fig. 3). The large effect of activated ras on gene expression in AtT20 cells appears restricted to a subset of expressed genes. The down-regulation of expression of the POMC gene may be by modulation of the activity of sequence-specific transcription factors or an accessory protein that interacts with the transactivation domain of a DNA-binding protein.

The proopiomelanocortin promoter is composed of a large number of synergistically interacting DNA elements (Therrien and Drouin, 1991). It is possible that one or more transcription factor proteins that bind to the POMC gene are inactivated or reduced in their expression as a result of transformation by the EJ-ras gene. We are currently making nuclear extracts from normal and ras-transformed AtT20 cells to examine the complement of binding activities to different cis-acting elements from the POMC promoter.

In studies of the effects of activated ras on fibroblast cell lines, ras transformation results in de-differentiation and increased cell proliferation (Barbacid, 1987). In contrast, PC12 cells, an adrenal chromaffin cell line, are induced to terminal differentiation by nerve growth factor or following differentiation to sympathetic neurons following nerve growth factor treatment. This differentiation can be inhibited or reversed by microinjection of the neutralizing anti-Ras monoclonal antibody Y13-259 (Hagag et al., 1986; Furth et al., 1982) thereby implicating a ras-dependent signaling pathway as a necessary component in the action of a neural differentiating agent.

We have reported here that a profound down-regulation in polypeptide hormone gene expression occurs in AtT20 cells stably transformed by EJ-ras. We recently reported on alterations in electrophysiological properties and the induction of two potassium channel mRNA species in ras-transformed cells (Flamm et al., 1990; Hemmick et al., 1992). The preprogrammed responses of PC12 cells and AtT20 cells to the intracellular second messenger pathway mediated by ras provide interesting models to study the regulation of differentiation by a neurotrophin-mediated intracellular signaling pathway common to neuroendocrine cells.

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