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Microarray and Suppression Subtractive Hybridization Analyses of Gene Expression in Pheochromocytoma Cells Reveal Pleiotropic Effects of Pituitary Adenylate Cyclase-Activating Polypeptide on Cell Proliferation, Survival, and Adhesion

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Pituitary adenylate cyclase-activating polypeptide (PACAP) exerts trophic effects on several neuronal, neuroendocrine, and endocrine cells. To gain insight into the pattern of the transcriptional modifications induced by PACAP during cell differentiation, we studied the effects of this neuropeptide on rat pheochromocytoma PC12 cells. We first analyzed the transcriptome of PC12 cells in comparison to that of terminally differentiated rat adrenomedullary chromaffin cells, using a high-density microarray, to identify genes associated with the proliferative phenotype that are possible targets of PACAP during differentiation of sympathoadrenal normal and tumoral cells. We then studied global gene expression in PC12 cells after 48 h of exposure to PACAP, using both cDNA microarray and suppression subtractive hybridization technologies. These complementary approaches resulted in the identification of 75 up-regulated and 70 down-regulated genes in PACAP-treated PC12 cells. Among the genes whose expression is modified in differentiated cells, a vast majority are involved in cell proliferation, survival, and adhesion/motility. Expression changes of most of these genes have been associated with progression of several neoplasms. A kinetic study of the effects of PACAP on some of the identified genes showed that the neuropeptide likely exerts early as well as late actions to achieve the gene expression program necessary for cell differentiation. In conclusion, the results of the present study underscore the pleiotropic role of PACAP in cell differentiation and provide important information on novel targets that could mediate the effects of this neuropeptide in normal and tumoral neuroendocrine cells. (*Endocrinology* 144: 2368–2379, 2003)

DIFFERENTIATION IS A FUNDAMENTAL process necessary for the specification of the various cell phenotypes during development and is a key step of cell growth that allows the transition from proliferating progenitor cells to specialized, functionally oriented cells. Understanding the molecular mechanisms underlying the numerous facets of cell differentiation for a given phenotype can be of utility for the study of not only developmental aspects but also tumorigenic events.

Elucidation of the genetic program that governs differentiation of a cell type can be approached by using *in vitro* models to gain insight into the molecular events occurring *in vivo*. The pheochromocytoma PC12 cell line, which originates from a tumor of rat adrenochromaffin cells, has been widely used to decipher the mechanisms of neuroendocrine and neuronal cell differentiation (1, 2). Adrenochromaffin cells are terminally differentiated neuroendocrine cells that derive from neural crest progenitors that also give rise to the sympathetic neurons. Differentiation of PC12 cells can be induced by different trophic factors, including nerve growth factor and glucocorticoids, toward sympathetic and chromaffin-like phenotypes (3, 4).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino acid, α-amidated peptide that regulates multiple functions in the central nervous system and in peripheral tissues via two types of G protein-coupled receptors: a PACAP selective receptor, PAC1-R, and two PACAP/vasoactive intestinal polypeptide mutual receptors, VPAC1-R and VPAC2-R (5–7). These receptors have been

Abbreviations: Basp1, brain abundant, membrane attached signal protein 1; CgB, chromogranin B; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gas1, growth arrest specific 1; Id3, inhibitor of DNA binding 3; Madh1, mothers against decapentaplegic homolog; Mcam, melanoma cell adhesion molecule; Mest, mesoderm specific transcript; PACAP, pituitary adenylate cyclase-activating polypeptide; Ptprr, protein tyrosine phosphatase receptor type R; Q-RT-PCR, quantitative RT-PCR; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; SSH, suppression subtractive hybridization.

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shown to activate different signal transduction pathways that recruit several protein kinases, such as protein kinase A and the MAPK ERK 1/2, which in turn induce or repress transcription of genes associated with homeostasis, growth, and differentiation in various cell types (6, 8–10). PACAP has been shown to induce growth arrest and to promote neuritic extension in PC12 cells (8, 11, 12), thus offering an opportunity to study the differentiation mechanisms induced by a ligand of G protein-coupled receptors in neuroendocrine cells.

In the adrenal medulla, PACAP has been shown to function as a neurotransmitter to regulate catecholamine, as well as neuropeptide biosynthesis and release *in vitro* and *in vivo*, through activation of PAC1-R and downstream signaling cascades in physiological and pathophysiological conditions (13–18). The presence of PAC1-R has also been demonstrated in pheochromocytomas by receptor autoradiography (19), indicating that PACAP may act on these tumors to influence catecholamine release *in vivo*, a life-threatening process in patients with pheochromocytoma. In fact, PACAP-like immunoreactivity has also been observed in pheochromocytomas (20), suggesting that an autocrine loop involving PACAP and its receptor may be responsible for a chronic effect of the neuropeptide in this type of tumor. In addition, PACAP may exert trophic and antiapoptotic effects that could influence the progression and differentiation of neoplastic cells, as has been reported in various neuroendocrine tumors (21–23).

In a recent study aimed at characterizing the phenotype of PACAP-differentiated PC12 cells, we have shown that the neuropeptide elicits a dual neuronal and neuroendocrine differentiation, suggesting that PACAP may represent a trophic factor for sympathoadrenal cells (24). PACAP altered the electrical properties and the expression of genes encoding noradrenergic-determining transcription factors as well as components of the secretory machinery in differentiated cells, indicating that the neuropeptide triggers the transcription of a wide variety of genes to induce cell differentiation. In the present study, we report on the global gene expression changes occurring in PC12 cells that have been differentiated for 48 h by PACAP, using both high-density microarray and suppression subtractive hybridization (SSH) technologies. In addition, validation of PACAP-regulated genes has been performed with a homemade macroarray and by Northern blot and quantitative RT-PCR analyses. These efforts have resulted in the identification of genes and gene families that are candidates for early and late molecular mechanisms underlying cell differentiation induced by PACAP that could occur in normal and tumoral conditions.

Materials and Methods

Animals and cell culture

Male Wistar rats (Centre d'Elevage Depré, Saint Doulchard, France) weighing 250–350 g were maintained under controlled conditions of temperature (22 C) under an established photoperiod (lights on from 0700 –1900 h). Rats had free access to laboratory chow (UAR, Epinay-sur-Orge, France) and water. All manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators. PACAP38 was synthesized by the solid phase methodology and purified by HPLC as previously described (25). The identity of the peptide was verified by mass spectrometry. Rat pheochromocytoma PC12 cells were purchased from the European Collection of Cell Culture (Salisbury, Wiltshire, UK). PC12 cells were originally derived from the New England Deaconess Hospital strain of Wistar rats that exhibited a markedly increased incidence of spontaneous pheochromocytoma (1, 26, 27). These cells were maintained in DMEM (Sigma-Aldrich Corp., Saint-Quentin Fallavier, France) supplemented with 10% horse serum (Invitrogen, Cergy Pontoise, France), 5% fetal bovine serum (BioWhittaker Europe, Verviers, Belgium), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich Corp.) at 37 C in 5% CO_2. The medium was renewed every 2–3 d. Twenty-four hours after plating, differentiation of PC12 cells was initiated by adding 100 nM PACAP38.

DNA microarrays, hybridization, and data analysis

A glass microarray containing over 15,000 mouse embryonic/placental cDNA probes (28) obtained from the National Institute on Aging (NIA) Mouse 15K cDNA library and corresponding to 15,264 Unigene clusters (for details, see http://lgsun.grc.nia.nih.gov/cDNA/15k.html) was used in this study. Targets were prepared from total RNA that was isolated from rat adrenal medulla or PC12 cells by the method of Chomczynski and Sacchi (29) using the Tri-Reagent (Sigma-Aldrich Corp.). The RNA samples were subsequently purified on RNeasy Mini Spin Columns (QIAGEN, Courtaboeuf, France) and quantified by spectrophotometry. Quality of the RNA was checked by ethidium bromide-staining of the 28S and 18S ribosomal RNA on a formaldehyde-agarose gel. Labeling and hybridization were performed according to standard National Human Genome Research Institute protocols (http://www.nhgri.nih.gov/UACORE/protocols.html). Briefly, 50–100 μg of purified RNA was reverse transcribed with Superscript II reverse transcriptase Rnase H^- (Life Technologies, Inc., Gaithersburg, MD) in the presence of either Cy5- or Cy3-deoxyuridine triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ) and oligo(deoxythymidine)$_{12–18}$ as previously described (30). Microarrays were hybridized with combined Cy5- and Cy3-labeled targets at 65 C overnight in a mix containing 2× Denhardt's solution, 3.2× saline sodium citrate (SSC), and 0.5% sodium dodecyl sulfate (SDS). The slides were washed at room temperature in 0.5× SSC, 0.1% SDS for 2 min; 0.5× SSC, 0.01% SDS for 2 min; and 0.06× SSC solution for 2 min. The arrays were then scanned (Agilent Technologies, Foster City, CA), and the measured intensities of the red and green fluorescent signals were normalized and filtered through quality control parameters and used to calculate gene expression ratios between the two targets using the IPLab software (Scanalytics, Fairfax, VA). Three independent experiments were performed for each comparative hybridization, and mean values were calculated. PACAP-regulated genes were resequenced, and the identity of the clones was confirmed and updated through comparison with sequences in National Center for Biotechnology Information (NCBI) databases using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Rat Genefilter microarrays GF300 and GF301 (Invitrogen) containing more than 10,000 sequence-verified rat cDNA probes obtained from the IMAGE Consortium (Laboratory Integrated Molecular Analysis of Genomes and their Expression, Lawrence Livermore National Laboratory, Livermore, CA) were also used in this study. Target cDNAs from untreated or PACAP-treated PC12 cells were prepared from total RNA after RT as described above, in the presence of [α-^{33}P]dCTP (Perkin-Elmer Corp., Courtaboeuf, France). The Genefilters were hybridized at 42 C with the ^{33}P-labeled targets in the MicroHyb solution (Invitrogen) supplemented with 1 μg/ml mouse Cot-1 DNA (Invitrogen), 1 μg/ml poly dA (Invitrogen), and 50 μg/ml yeast tRNA (Sigma-Aldrich Corp.). The membranes were washed four times in 2× SSC, 0.1% SDS at room temperature, and twice in 0.1× SSC, 0.1% SDS for 15 min at 50 C. Target cDNAs from each cell condition were simultaneously hybridized to two different Genefilters of the same type, and each Genefilter was then stripped and rehybridized with the opposite target cDNAs to avoid system variability that may be associated with the use of different filters. Images of hybridized Genefilters were obtained using a STORM phosphor imager (Amersham Pharmacia Biotech). The signal intensities of the hybridized probes in a Genefilter were normalized to those of 384 control probes (actin and genomic DNA) that are printed at various areas of the filter, and the consistency of the standardized values in the different hybridizations was assessed by the Pathways 4 software (Invitrogen) before the calculation of gene expression ratios.

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SSH

Total RNA was extracted from undifferentiated and PACAP-differentiated PC12 cells as described above, and poly(A)$^+$ RNA was isolated with the PolyATtract mRNA Isolation System (Promega Corp., Charbonnières, France). cDNAs were synthesized from 2 μg of poly(A)$^+$ RNA, and subtractive hybridization was performed using the PCR-Select cDNA subtraction kit (BD Biosciences, Saint-Quentin en Yvelines, France). To isolate PACAP-induced transcripts, cDNAs from PACAP-treated cells were ligated to oligonucleotide linkers and hybridized with excess cDNAs from untreated cells. After hybridization, differentially expressed transcripts were selectively amplified by suppression PCR (31). Amplified cDNAs were introduced into the pCR4-TOPO vector (Invitrogen) and electroporated into DH10B cells to generate a subtractive library. This library was plated, and the plasmids of bacterial lifts were screened to eliminate false positive clones. Briefly, RNA derived from undifferentiated and PACAP-differentiated PC12 cells was reverse transcribed, as described above, in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech) and used to sequentially hybridize the bacterial lifts at 42 C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's, 200 μg/ml salmon sperm DNA, 50 μg/ml yeast tRNA, 0.1% SDS, and 50 mM phosphate buffer (pH 6.5). The membranes were washed four times in 2× SSC, 0.1% SDS at room temperature, and twice in 0.1× SSC, 0.1% SDS for 15 min at 50 C. Filters were analyzed using the STORM phosphor imager system, and the images corresponding to hybridization with differentiated or undifferentiated PC12 targets were compared using the Z3 software (Compugen, Jamesburg, NJ) to identify PACAP-regulated clones. Positive clones were identified through sequencing and comparison with sequences in NCBI databases using the BLAST software.

Macroarray preparation and hybridization

Clones identified by microarray and subtractive hybridization analyses were amplified with universal primers and the DyNAzyme EXT DNA polymerase, following the instructions of the manufacturer (Ozyme, Saint-Quentin en Yvelines, France), in a PCRexpress thermal cycler (Hybaid, Paris, France) and used as probes to make a macroarray. The quality of the amplified DNA was checked by migration on a 1% agarose gel. The PCR products contained in a 384-well plate were directly printed on Hybond NX membranes (Amersham Pharmacia Biotech) using a ChipWriter system (Virtek, Waterloo, Canada). These filters were denatured with a 0.4 M NaOH, 0.1 M NaCl solution for 5 min and neutralized with a 40 mM Na_2HPO_4/NaH_2PO_4 solution (pH 7.2) for 5 min. The macroarrays were hybridized with target cDNAs derived from untreated or PACAP-treated PC12 cells as described above for the Genefilters. Images of the hybridized macroarrays obtained from the phosphor imager were quantified with the XDotsReader software (Cose, Dugny, France). Hybridization signals were normalized to those of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe that was printed at several locations of the macroarray.

Northern blot analysis

Total RNA was prepared as described above, dissolved in denaturing buffer, heated at 65 C for 15 min, and fractionated on formaldehyde-agarose gels. After staining with ethidium bromide, gels were blotted on Hybond NX membranes (Amersham Pharmacia Biotech) and fixed by UV irradiation. The filters were subsequently hybridized at 42 C with ^{32}P-labeled random primed (Prime-a-Gene Labeling System, Promega Corp.) fragments of inhibitor of DNA binding 3 (Id3), mesoderm specific transcript (Mest), melanoma cell adhesion molecule (Mcam), growth arrest specific 1 (Gas1), chromogranin B (CgB), and brain abundant, membrane-attached signal protein 1 (Basp1) cDNAs in a solution containing 40–50% formamide, 5× SSC, 5× Denhardt's, 200 μg/ml salmon sperm DNA, 0.1% SDS, 50 mM phosphate buffer (pH 6.5). The membranes were washed as described above for the Genefilters or the macroarrays. Filters were analyzed by using the STORM phosphor imager and the ImageQuant 5.1 software (Amersham Pharmacia Biotech). RNA loading variations were corrected by scanning the ethidium bromide-stained ribosomal RNA using the DensyLab 2.0.5 software (Bioprobe Systems, Montreuil, France).

Quantitative RT-PCR (Q-RT-PCR)

Approximately 1 μg of total RNA extracted as described above was submitted to DNase I (Rnase-free; Promega Corp.) digestion and reverse transcribed using random hexamers pdN_6 (Amersham Pharmacia Biotech) and SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). Gene-specific forward and reverse primers were chosen using the Primer Express 2 software (PE Applied Biosystems, Courtaboeuf, France) as follows: 5′-AACTCCCTCAAGATTGTCAGCAA-3′ and 5′-GTGGTCATGAGCCCTTCCA-3′ for GAPDH; 5′-GCGACACATCGGGAAAGG-3′ and 5′-TCGACTCTGCACGAAGATGCT-3′ for mothers against decapentaplegic homolog 1 (Madh1); 5′-CAGTTGAAAGAAGAAGGAGTCGTAGA-3′ and 5′-AATTCATACTGCTCACTGGTTTGGA-3′ for protein tyrosine phosphatase receptor type R (Ptprr). Real-time PCR (Q-RT-PCR) was performed in a premade reaction mix (PE Applied Biosystems) in the presence of the transcribed cDNA and 300 nM of specific primers, using the SYBR green chemistry and an ABI Prism 7000 (PE Applied Biosystems). Relative amounts of Madh1 and Ptprr mRNAs were determined from a standard curve generated using different dilutions of the cDNA and by normalizing against a non-variable control gene, GAPDH, that was analyzed in parallel on the same RT.

Results and Discussion

Global analysis revealed differentially expressed genes associated with proliferating pheochromocytoma cells

Because the rat pheochromocytoma PC12 cell line has been originally derived from adrenomedullary chromaffin cells (1, 26), we first compared the transcriptomes of PC12 cells and rat adrenomedullary cells to better understand the effect of PACAP on tumoral cell proliferation and differentiation. We predicted that several genes important for cell growth, survival, and adhesion/motility in this lineage would be identified that could be regulated by trophic factors during differentiation of sympathoadrenal-derived normal or tumoral cells. Gene expression changes between PC12 cells and chromaffin cells were assessed by using a microarray derived from the NIA 15K mouse embryonic cDNA library (28). A mouse microarray was used in this study because a similar rat developmental array was not available. We anticipated that the mouse array made from an embryonic cDNA library could be very useful to identify genes that would be regulated by trophic factors during cell differentiation. Of the 15,264 genes analyzed, 1,048 were differentially expressed by at least 2-fold between the tumoral and normal adrenomedullary chromaffin cells in three independent experiments, using three different RNA preparations from different animals or cell cultures, and three separate hybridizations. The majority of these genes (71.4%) were more highly expressed in PC12 cells compared with adrenomedullary cells, and about 50% were unnamed genes. Because of space limitation, we have arbitrarily chosen to present in this report only named genes that showed a fold change of 2.5 or more (Tables 1 and 2). These genes were classified in functional categories using the Onto-Express V.2 software (32) based on the LocusLink database in NCBI.

Not surprisingly, a vast majority of the named genes that were found to be differentially expressed between PC12 and rat adrenochromaffin cells are associated with proliferation and DNA replication (Tables 1 and 2). In particular, numerous regulators of cell cycle progression were highly expressed in PC12 cells. These include several members of the hexameric mini chromosome maintenance deficient MCM2–7 complex that is required for the onset of the S phase

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TABLE 1. Named genes overexpressed in PC12 cells *vs.* rat adrenomedullary cells

Gene	Ratio	Acc.	Unigene
Proliferation/DNA synthesis			
Mini chromosome maintenance deficient 5	19.7 ± 13.8	AW536273	Mm.5048
Cell division cycle 2 homolog A	11.8 ± 5.2	AW536234	Mm.4761
Polymyositis/scleroderma autoantigen 1	9.7 ± 4.5	AU014747	Mm.116711
Mini chromosome maintenance deficient 6	8.1 ± 3.2	AW555913	Mm.4933
Cyclin A2	7.3 ± 3.8	AU020259	Mm.4189
Cyclin B2	5.6 ± 2.0	AU045362	Mm.22592
Nuclear protein 95	4.8 ± 2.3	AW536640	Mm.42196
Calcium binding protein A6 (calcyclin)	4.6 ± 1.1	AU016154	Mm.100144
Calcyclin binding protein	4.6 ± 0.3	AU020539	Mm.10702
DNA primase, p49 subunit	4.4 ± 0.7	AW552518	Mm.2903
Nucleophosmin 1	4.3 ± 0.3	AW553526	Mm.6343
Mini chromosome maintenance deficient	4.2 ± 2.1	AW536712	Mm.4502
Cyclin E2	4.2 ± 0.6	AW544792	Mm.35867
Microspherule protein 1	3.9 ± 1.4	AU043702	Mm.3716
Pituitary tumor-transforming 1	3.9 ± 1.4	AW555095	Mm.6856
Mini chromosome maintenance deficient 2	3.7 ± 0.9	AW553939	Mm.16711
Casein kinase 1, epsilon	3.5 ± 0.7	C87299	Mm.30199
Ligase I, DNA, ATP-dependent	3.4 ± 0.2	C77364	Mm.1013
RAN, member RAS oncogene family	3.4 ± 0.3	AW557767	Mm.7521
Polymerase, gamma	3.2 ± 0.4	AW536165	Mm.3616
H1 histone family, member 0	3.2 ± 0.6	Mm.24350	AU042799
H2A histone family, member Z	3.2 ± 0.0	Mm.36705	AW539814
Prothymosin alpha	3.2 ± 0.8	AW548086	Mm.19187
Thymopoietin	3.1 ± 0.9	AW550700	Mm.124
Checkpoint kinase 1 homolog	3.1 ± 0.6	C78961	Mm.16753
Mini chromosome maintenance deficient 7	3.1 ± 0.2	AW554120	Mm.18923
Growth associated protein 43	3.0 ± 1.1	AW558609	Mm.1222
Polymerase (DNA directed), delta 2, regulatory subunit (50 kDa)	3.0 ± 0.1	AW537005	Mm.35768
DNA methyltransferase 3A	3.0 ± 1.4	AW549977	Mm.5001
Cyclin-dependent kinase 2-associated protein 1	2.9 ± 0.3	AW549716	Mm.196624
Cyclin B1, related sequence 1	2.9 ± 0.2	AU045643	Mm.22569
Cyclin-dependent kinase 2	2.8 ± 0.1	AW547935	Mm.118
Ribonucleotide reductase M1	2.8 ± 0.7	AW536068	Mm.656
Nucleosome assembly protein 1-like 1	2.8 ± 1.2	Mm.3797	AU018118
Wee 1 homolog	2.8 ± 0.3	AW559064	Mm.8108
Translin	2.8 ± 0.6	Mm.14644	AW545280
Thymidine kinase 1	2.7 ± 0.3	AW544533	Mm.2661
Casein kinase II, alpha 2, polypeptide	2.7 ± 0.4	AU019476	Mm.28881
Inosine 5'-phosphate dehydrogenase 2	2.7 ± 0.1	AW548016	Mm.6065
Topoisomerase (DNA) II alpha	2.6 ± 0.5	Mm.4237	AW554229
Proliferation-associated protein 1	2.6 ± 0.4	AW536189	Mm.4742
B-cell translocation gene 2	2.6 ± 0.1	C87946	Mm.903
SET translocation	2.5 ± 0.2	AW537968	Mm.28805
Transcription factors and regulators			
High mobility group box 2	10.0 ± 2.4	AW546306	Mm.1693
High mobility group box 1	7.3 ± 3.8	AW536666	Mm.16421
Y box protein 3	4.0 ± 0.2	C77087	Mm.193526
Cold shock domain protein A	3.8 ± 0.3	AW552638	Mm.141567
Hypoxia inducible factor 1, alpha subunit	3.6 ± 0.9	AW543477	Mm.3879
Embryonic lethal, abnormal vision-like 2 (Hu antigen B)	3.5 ± 1.3	AU016075	Mm.3823
Butyrate response factor 2	3.2 ± 1.0	AW543115	Mm.28161
Histone acetyltransferase	3.0 ± 1.1	C85086	Mm.30996
Melanoma antigen, family D, 1	3.0 ± 1.2	AW552736	Mm.27578
Runt related transcription factor 2	2.9 ± 0.3	C85431	Mm.4509
Ewing sarcoma homolog	2.7 ± 0.8	AW556031	Mm.142822
Zinc finger protein 207	2.6 ± 0.3	AW556214	Mm.12236
GATA-binding protein 2	2.6 ± 0.2	AW538547	Mm.1391
Transcription factor E2a	2.5 ± 0.5	AW553936	Mm.3406
RNA processing			
Quaking	3.8 ± 0.6	AU023418	Mm.2655
NS1-associated protein 1	3.5 ± 1.0	AW544805	Mm.27972
Heterogeneous nuclear ribonucleoprotein A1	3.4 ± 0.4	AW546008	Mm.27927
RNA and export factor binding protein 1	3.1 ± 0.3	AW551815	Mm.1886
Polypyrimidine tract binding protein	3.1 ± 0.7	AW538624	Mm.19117
RNA binding motif protein 3	2.8 ± 0.1	AW538776	Mm.2591
U5 small nuclear ribonucleoprotein 116 kDa	2.7 ± 0.7	AW547534	Mm.673
Ribonucleic acid binding protein S1	2.6 ± 0.2	AW545556	Mm.1951
Non-POU-domain-containing, octamer-binding protein	2.5 ± 0.6	AW556707	Mm.21559
Protein processing/apoptosis			
Heat shock protein, 25 kDa	5.5 ± 0.3	AU021579	Mm.13849
Ubiquitination factor E4B	4.2 ± 1.4	C85947	Mm.21634
Crystallin, alpha C	3.6 ± 0.6	AU018999	Mm.21549
Peroxiredoxin 4	3.5 ± 0.6	AW555794	Mm.19127
DnaJ (Hsp40) homolog, subfamily B, member 6	3.5 ± 1.4	AA408610	Mm.2701
Nucleoredoxin	3.3 ± 0.2	AU041190	Mm.27915
Ubiquitin specific protease 14	3.3 ± 0.5	AU020664	Mm.25149
26S proteasome-associated pad1 homolog	3.2 ± 0.1	AW538992	Mm.27933
EGL nine homolog 1	3.1 ± 0.1	AW555687	Mm.140619
G elongation factor	3.1 ± 0.2	C76703	Mm.27288
Proteasome (prosome, macropain) 26S subunit, ATPase 3	2.8 ± 0.3	AA409481	Mm.20946
Chaperonin subunit 3 (gamma)	2.8 ± 0.0	AW556780	Mm.3576
Heat shock protein, 84 kDa 1	2.8 ± 0.6	AW556206	Mm.2160
Eukaryotic translation initiation factor 3, subunit 8 (110 kDa)	2.7 ± 0.7	AW552427	Mm.22776
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	2.6 ± 0.2	AW536390	Mm.29760
BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	2.6 ± 0.4	AW553554	Mm.2159
Protease (prosome, macropain) 26S subunit, ATPase 5	2.6 ± 0.2	C76006	Mm.865
Chaperonin subunit 4 (delta)	2.5 ± 0.1	AW536843	Mm.6821
Eukaryotic translation initiation factor 4, gamma 2	2.5 ± 0.6	AW536743	Mm.525
Signaling			
Rac GTPase-activating protein 1	13.1 ± 3.2	AU042813	Mm.27141
Leukemia-associated gene (stathmin 1)	6.6 ± 1.8	AW549685	Mm.28479
Calcium binding protein, 39 kDa	6.2 ± 5.1	AW551261	Mm.21684
Shc SH2-domain binding protein 1	4.8 ± 0.7	C87245	Mm.37601
Interleukin enhancer binding factor 3	4.8 ± 2.2	AW536403	Mm.20935
Serine/threonine kinase 5	4.6 ± 1.3	AA410169	Mm.3468
RAN binding protein 1	4.2 ± 0.3	AA409179	Mm.3752
RAN GTPase activating protein 1	4.1 ± 0.6	AW536168	Mm.3833
Homer, neuronal immediate early gene, 2	4.1 ± 1.0	AA407944	Mm.228
Trk-fused gene	4.1 ± 0.3	AW556895	Mm.41374
Mitogen activated protein kinase kinase 2	3.8 ± 2.5	C78431	Mm.196584
Regulator of G-protein signaling 17	3.5 ± 0.9	AU044873	Mm.44606
Macrophage migration inhibitory factor	3.4 ± 1.0	AW544543	Mm.2326
Phospholipase c neighboring	3.1 ± 0.3	C86296	Mm.140
Guanine nucleotide binding protein, gamma 3 subunit	2.9 ± 1.0	C87570	Mm.27307
Maternal embryonic leucine zipper kinase	2.9 ± 0.5	AW537856	Mm.4674
Immediate early response, erythropoietin 4	2.8 ± 0.2	AW537102	Mm.27804
Interleukin enhancer binding factor 2	2.7 ± 0.3	AW537207	Mm.21534
Cyclic AMP phosphoprotein, 19kD	2.7 ± 0.5	AU019153	Mm.30728
Calumenin	2.6 ± 0.8	AW556006	Mm.7515
Signal recognition particle receptor, B subunit	2.6 ± 0.1	AW536301	Mm.7568
Cytoskeleton			
Keratin complex 2, basic, gene 8	12.4 ± 0.3	AW542449	Mm.6800
Tuba4 Tubulin, alpha 4	7.5 ± 1.5	AA408725	Mm.1155
Tubulin, beta 5	7.1 ± 1.3	AW542954	Mm.1703
Kinesin-like 5	4.2 ± 0.5	AU022593	Mm.28386
Keratin complex 1, acidic, gene 18	3.8 ± 0.4	AW536107	Mm.22479
Lamin A	3.8 ± 0.8	AW544095	Mm.3436
Transgelin 2	3.5 ± 0.6	AW544177	Mm.22632
Septin 8	3.1 ± 0.5	AW547569	Mm.44604
Lamin B1	3.1 ± 1.4	AW542456	Mm.4846
Alpha actinin 4	2.7 ± 0.5	AW552978	Mm.143830
Cell matrix/adhesion			
Galectin-3	4.4 ± 0.4	AW543680	Mm.2970
Syndecan 1	4.2 ± 2.5	AW540939	Mm.2580
Galectin-1	4.1 ± 0.8	AA410090	Mm.43831
Embigin	3.8 ± 0.7	AW536236	Mm.89123
Roundabout homolog 1	2.7 ± 1.3	AW537036	Mm.20832
Claudin 3	2.7 ± 0.3	AU040223	Mm.28921
Ion pumps/transporters			
Monocarboxylate transporter 4	7.8 ± 2.6	AW536253	Mm.28632
Proteolipid protein 2	4.3 ± 0.9	AW536141	Mm.18565
Potassium interm./small conduct. ca-activated channel, subf. N, 4	4.2 ± 0.6	C86468	Mm.9911
ATPase, H+ transporting, lysosomal, beta 56/58 kDa, isoform 2	3.8 ± 0.6	AW539631	Mm.10727
Calcium channel beta 3 subunit	3.0 ± 0.5	AW547609	Mm.3544
Flavin containing monooxygenase 5	2.7 ± 0.3	AW547363	Mm.1668
Solute carrier family 2 (facilitated glucose transporter), member 1	2.6 ± 0.5	AW543423	Mm.21002
Heme oxygenase (decycling) 1	2.6 ± 0.4	AW544501	Mm.17960
Vesicle/protein trafficking			
Karyopherin (importin) alpha 2	6.6 ± 0.5	C79184	Mm.12508
Importin beta	3.7 ± 1.3	AU020544	Mm.16710
RAS-like, family 2, locus 9	3.7 ± 0.2	AU044042	Mm.103632
Vertebrate homolog of C. elegans Lin-7 type 3	3.2 ± 1.7	C85192	Mm.218854
Karyopherin (importin) alpha 3	2.5 ± 0.4	AU023086	Mm.25548
Metabolism			
Lactate dehydrogenase 1, A chain	6.3 ± 1.4	AW536162	Mm.141443
Asparagine synthetase	4.5 ± 0.3	AW557889	Mm.2942
Phosphofructokinase, liver, B-type	4.0 ± 0.4	AW536420	Mm.1166
3-phosphoglycerate dehydrogenase	3.7 ± 0.6	C79697	Mm.16898
Pyruvate kinase 3	3.3 ± 0.4	AW537401	Mm.2635
Phosphoribosyl pyrophosphate synthetase 1	3.3 ± 0.2	AW543694	Mm.27454
Uridine-cytidine kinase 2	3.2 ± 0.8	AW543081	Mm.25309
Glucose phosphate isomerase 1 complex	3.2 ± 0.3	AW550689	Mm.589
Ornithine decarboxylase antizyme inhibitor	3.2 ± 0.8	AW558751	Mm.6775
Enolase 1, alpha non-neuron	3.2 ± 0.2	AW536817	Mm.90587
Fatty acid synthase	2.7 ± 0.4	AW552727	Mm.3760
Thymidine kinase 1	2.7 ± 0.3	AW544533	Mm.2661
Citrate synthase	2.5 ± 0.7	AU024674	Mm.43822
Miscellaneous			
Mesoderm specific transcript	15.3 ± 2.5	AW553763	Mm.1089
Brain abundant, membrane attached signal protein 1	8.1 ± 1.0	AA408949	Mm.29586
Reduced expression 3	4.4 ± 0.3	AW536404	Mm.14768
Olfactomedin related ER localized protein	3.9 ± 1.2	AW548326	Mm.43278
Quaking	3.8 ± 0.6	AU023418	Mm.2655
CD24a antigen	3.0 ± 0.3	AU042170	Mm.6417
Serologically defined colon cancer antigen 33	2.9 ± 0.5	AU023609	Mm.102136
Timeless homolog	2.8 ± 0.8	AW545411	Mm.6458
Membrane bound C2 domain containing protein	2.7 ± 0.1	AW545598	Mm.29010
Selectin, endothelial cell, ligand	2.7 ± 0.7	AW552445	Mm.488
Hematological and neurological expressed sequence 1	2.6 ± 0.3	AW555684	Mm.1775
Seb4-like	2.6 ± 0.1	AU043434	Mm.3865
Feminization 1 b homolog	2.6 ± 0.8	AU018354	Mm.24069

The GenBank accession no. (Acc.) and the Unigene cluster for each gene are indicated. The ratios ± SEM were determined from three different experiments.

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TABLE 2. Named genes less expressed in PC12 cells *vs.* rat adrenomedullary cells

Gene	Ratio	Acc.	Unigene
Proliferation/DNA synthesis			
cysteine rich intestinal protein	0.31 ± 0.02	AU042095	Mm.22049
Thymidylate synthase	0.28 ± 0.18	AW546106	Mm.5879
Cyclin D2	0.26 ± 0.06	AW557306	Mm.3141
Cyclin-dependent kinase inhibitor 1C (P57)	0.20 ± 0.04	AU040767	Mm.168789
Transcription factors and regulators			
E26 avian leukemia oncogene 2, 3' domain	0.35 ± 0.10	AA407887	Mm.22365
Myeloid ecotropic viral integration site-related gene 1	0.29 ± 0.06	AW553445	Mm.31436
Nuclear receptor subfamily 5, group A, member 1	0.20 ± 0.06	C85959	Mm.31387
Ets variant gene 6 (TEL oncogene)	0.15 ± 0.01	AW546695	Mm.2017
Butyrate response factor 1	0.10 ± 0.04	AW551822	Mm.18571
RNA processing			
Splicing factor, arginine/serine-rich 5	0.37 ± 0.02	AW549360	Mm.43331
Heterogeneous nuclear ribonucleoprotein C	0.21 ± 0.04	C81083	Mm.25074
Protein processing/apoptosis			
Protease (prosome, macropain) 28 subunit, alpha	0.39 ± 0.02	AW551192	Mm.830
Serine (or cysteine) proteinase inhibitor, clade H (hsp 47), member	0.37 ± 0.11	AW550653	Mm.22708
Glutathione peroxidase 4	0.35 ± 0.02	AW546594	Mm.2400
Proprotein convertase subtilisin/kexin type 5	0.33 ± 0.07	AU021857	Mm.3401
Glutathione peroxidase 1	0.33 ± 0.02	AW536643	Mm.1090
Heat shock 10 kDa protein 1 (chaperonin 10)	0.31 ± 0.03	AA409440	Mm.12970
Glutathione peroxidase 3	0.19 ± 0.09	AW550656	Mm.7156
Procollagen C-proteinase enhancer protein	0.19 ± 0.01	AW554530	Mm.18808
Peroxiredoxin 3	0.17 ± 0.01	AW554565	Mm.29821
Scavenger receptor class B1	0.13 ± 0.02	AW539410	Mm.4603
Signaling			
Lymphocyte antigen 6 complex, locus E	0.39 ± 0.13	AW536803	Mm.788
Transmembrane 7 superfamily member 1	0.38 ± 0.01	AW546472	Mm.1585
Prolactin receptor	0.37 ± 0.09	AU018948	Mm.2752
Regulator of G-protein signalling 10	0.35 ± 0.09	AU044521	Mm.18635
Delta-like homolog 1	0.29 ± 0.02	AA407811	Mm.157069
Membrane interacting protein of RGS16	0.28 ± 0.03	AU044044	Mm.30126
Pleiotrophin	0.25 ± 0.03	AW550271	Mm.3063
Regulator of G-protein signaling 2	0.25 ± 0.04	AU023169	Mm.28262
Eph receptor B6	0.22 ± 0.05	AU041627	Mm.1480
Melanocortin 2 receptor	0.13 ± 0.00	AW548422	Mm.41498
Benzodiazepine receptor, peripheral	0.10 ± 0.02	AA406181	Mm.1508
Cytoskeleton			
Synapsin I	0.35 ± 0.04	AW536982	Mm.196611
Cytoskeleton (continued)			
Four and a half LIM domains 1	0.34 ± 0.14	AW554915	Mm.3126
Myosin Vb	0.17 ± 0.08	AW546331	Mm.3536
Thymosin, beta 4, X chromosome	0.08 ± 0.02	AW555204	Mm.142729
Cell matrix/adhesion			
Biglycan	0.40 ± 0.05	AW551689	Mm.2606
Col3a1 Procollagen, type III, alpha 1	0.39 ± 0.06	AU017502	Mm.147387
CD9 antigen	0.33 ± 0.04	AW554302	Mm.2956
Microfibrillar-associated protein 2	0.28 ± 0.06	AW553505	Mm.7386
Milk fat globule-EGF factor 8 protein	0.25 ± 0.05	AW551325	Mm.1451
Procollagen, type IV, alpha 1	0.25 ± 0.06	AW559158	Mm.738
Decorin	0.11 ± 0.05	AW556372	Mm.1987
Tissue inhibitor of metalloproteinase 3	0.11 ± 0.04	AU042249	Mm.4871
Secreted phosphoprotein 1	0.06 ± 0.01	AU021551	Mm.321
Ion pumps/transporters			
Cytochrome b-561	0.38 ± 0.07	AW548509	Mm.154456
FXYD domain-containing ion transport regulator 3	0.33 ± 0.02	C86807	Mm.1662
Cytochrome P450, 1b1, benz[a]anthracene inducible	0.32 ± 0.14	C85808	Mm.4443
Selenoprotein P, plasma, 1	0.27 ± 0.12	AW557404	Mm.22699
Hemoglobin, beta adult major chain	0.19 ± 0.02	AW548342	Mm.142368
Hemoglobin alpha, adult chain 1	0.18 ± 0.09	AW549905	Mm.196110
Ferredoxin reductase	0.09 ± 0.02	AW556320	Mm.4719
Cytochrome P450, 11a, cholesterol side chain cleavage	0.04 ± 0.01	AU018054	Mm.108678
Metabolism			
Cytosolic cysteine dioxygenase 1	0.38 ± 0.06	AU021886	Mm.29996
Branched chain ketoacid dehydrogenase E1, alpha polypeptide	0.34 ± 0.03	AW553527	Mm.25848
Aminolevulinic acid synthase 1	0.32 ± 0.10	AU042924	Mm.19143
Retinal short-chain dehydrogenase/reductase 1	0.31 ± 0.08	AW549367	Mm.14063
Sterol O-acyltransferase 1	0.27 ± 0.03	AW558669	Mm.28099
Hydroxysteroid dehydrogenase-4, delta<5>-3-beta	0.16 ± 0.12	AW546473	Mm.14309
Spermidine/spermine N1-acetyl transferase	0.08 ± 0.02	AW546597	Mm.2734
Fibroblast growth factor regulated protein	0.06 ± 0.01	C77965	Mm.5378
Tyrosine hydroxylase	0.06 ± 0.01	C85951	Mm.1292
Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	0.05 ± 0.01	AU024029	Mm.140811
Miscellaneous			
Lipocalin 7	0.37 ± 0.10	AW553913	Mm.15801
Complement component 1, r subcomponent	0.35 ± 0.17	AW553572	Mm.24276
Calmin	0.32 ± 0.04	AW548197	Mm.89607
Complement component 1, q subcomponent, beta polypeptide	0.24 ± 0.03	AW555781	Mm.2570
Adipose differentiation related protein	0.15 ± 0.03	AW555596	Mm.381

The GenBank accession no. (Acc.) and the Unigene cluster for each gene are indicated. The ratios ± SEM were determined from three different experiments.

and DNA replication, as well as different cyclins and associated proteins. Of note is the lower expression of cyclin D2 in PC12 compared with adrenomedullary chromaffin cells, in accordance with reports showing an increased expression of this cyclin in certain growth-arrest conditions (33). Several other proteins that interact with components of the cell cycle, including pituitary tumor-transforming 1, prothymosin α, and thymopoietin, which are implicated in the proliferation of different tumoral cell types (34–36), also exhibited a higher expression in PC12 cells compared with nontumoral chromaffin cells.

The mRNA of various signaling proteins, *e.g.* GTPases and related proteins, were present at higher levels in PC12 cells, whereas those of hormonal receptors, *e.g.* prolactin or melanocortin receptors, were found at higher levels in adrenochromaffin cells. In addition, the expression of several transcription regulators involved in proliferation/differentiation mechanisms, such as members of the high-mobility group protein family, butyrate response factors or GATA-binding proteins, was also altered.

A large group of genes encoding protein processing and apoptosis factors showed a marked expression difference between proliferating PC12 cells and differentiated adrenochromaffin cells (Tables 1 and 2). In this group, several genes encoding ubiquitination and proteasomal factors were more intensely expressed in PC12 cells, reflecting a higher rate of protein degradation in the tumoral cells. On the contrary, genes implicated in protection from oxidative stress, such as glutathione peroxidases, were more highly expressed in chromaffin cells. It should be noted that genes encoding ion transporters like the potassium intermediate/small conductance calcium-activated channel or the selenoprotein P, which were differentially expressed between PC12 and adrenochromaffin cells, may also play a role in cell survival and protection of these cells.

Cytoskeleton and cell matrix/adhesion proteins are involved in a variety of biological responses including remodeling of cell morphology, cell-cell interactions, and cell motility. In this respect, the marked difference in mRNA levels of thymosin-β 4, an actin-modulating cytoskeletal protein whose expression is related to cell differentiation (37), is especially interesting. Important changes in the expression of numerous matrix and adhesion proteins including galectins, syndecan 1, and embigin, which are implicated in development, cell growth, apoptosis, and differentiation, were observed between PC12 and adrenochromaffin cells. Variations in the expression of these proteins are known to be associated with the aggressiveness and invasiveness of different types of tumors (38–40).

Although not exhaustive, this comparison of the transcriptomes of PC12 and adrenochromaffin cells provides insights into the genes and gene families whose expression is specifically altered in pheochromocytoma cells, many of which have not previously been described in these cells. The genes

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identified could represent molecular targets for trophic factors like PACAP to regulate different aspects of growth, survival, and adhesion/motility in physiological and pathophysiological conditions.

Analysis of PACAP-regulated genes in PC12 cells

Treatment of PC12 cells with 10^{-7} M PACAP for 48 h induced a profound morphological transformation with the appearance of numerous neuritic extensions (Fig. 1). Previous studies using similar conditions have shown that PACAP completely suppresses PC12 cell proliferation (41), suggesting that prolonged treatment with PACAP causes growth cessation of the majority of proliferating PC12 cells. We have previously shown that PACAP (10^{-7} M, 72 h) elicits a dual neuronal and neuroendocrine phenotype as assessed by the effect of the neuropeptide on noradrenergic-specifying transcription factors, cell excitability, and neurotransmitter storage and release machinery in PC12 cells (24). Such actions imply the regulation of a complex program of gene expression during differentiation of PC12 cells.

To determine the molecular events associated with the effect of PACAP on PC12 cells, we compared the transcriptome of cells treated with the neuropeptide for 48 h with that of untreated cells. We hypothesized that, after 2 d, it would be possible to detect changes in the expression of early as well as late genes that could be involved in PC12 cell differentiation under PACAP treatment. To obtain an overall view of the actions of PACAP on PC12 cell gene expression, we used the complementary approaches of high-density microarrays and SSH. We performed microarray gene analysis on the NIA 15K mouse embryonic cDNA library that was used to compare PC12 and chromaffin cell transcriptomes and also on rat random cDNAs from the IMAGE collection that should basically broaden our analysis. We also performed a direct comparison of PC12 cell mRNA populations in PACAP-treated (48 h) and untreated cells using the SSH technique. The rat microarray and SSH should also help to identify PACAP-regulated genes that may fail to hybridize with mouse clones of the NIA library for lack of sufficient sequence homology.

Microarray data analyses were performed by using an average fold change, derived from two independent experiments, of 1.5 or greater and excluding clones that exhibited an incoherent value in any of the different experiments. The ratio limit of 1.5 was used because gene expression changes in this range could be validated in this study by homemade macroarray, Northern blot, and Q-RT-PCR analyses (Fig. 2).

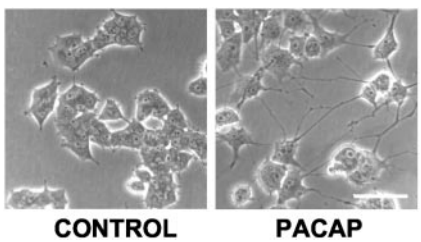

FIG. 1. Effect of PACAP on PC12 cells. Cells were plated at a density of 5×10^5 cells/ml and cultured for 1 d before treatment. The cells were left untreated (Control) or were treated with PACAP (100 nM, 48 h). *Scale bar*, 50 μm.

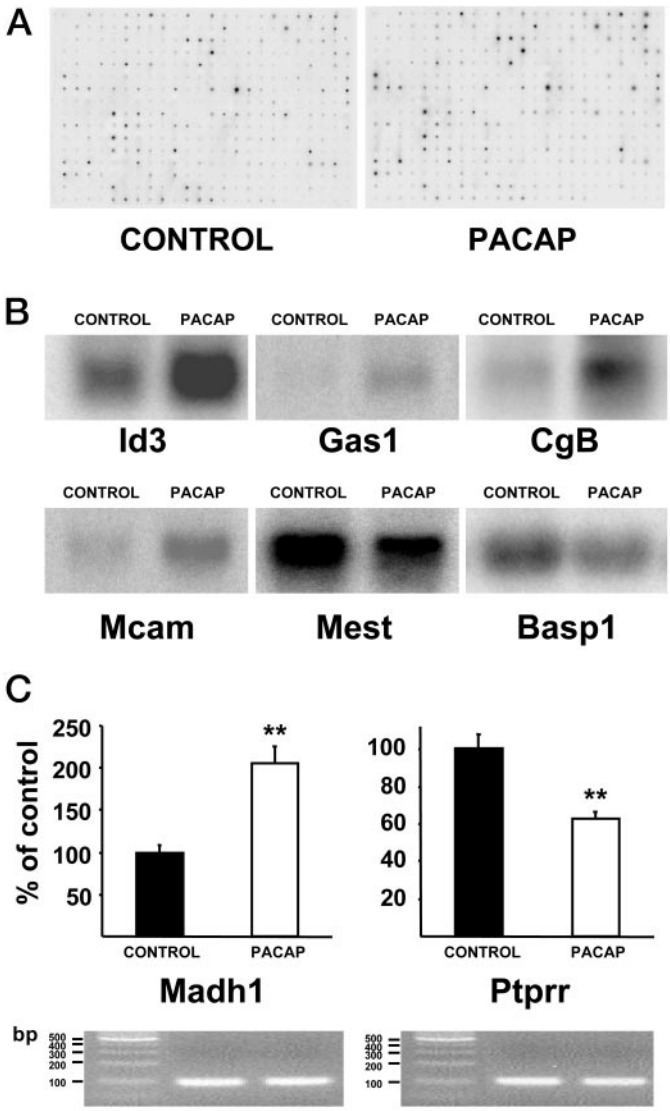

FIG. 2. Macroarray, Northern blot, and Q-RT-PCR analyses of PACAP-regulated genes in differentiated PC12 cells. A, RNA from untreated (Control) and PACAP-treated (100 nM, 48 h) PC12 cells was reverse transcribed in the presence of ^{33}P-dCTP and used to hybridize macroarrays containing cDNA probes derived from the NIA microarray and SSH-based library. B, Twenty μg of total RNA from control and PACAP-differentiated (100 nM, 48 h) PC12 cells was analyzed by Northern blot using specific ^{32}P-labeled cDNA probes for Id3, Gas1, CgB, Mcam, Mest, and Basp1. C, Q-RT-PCR analysis of Madh1 and Ptprr gene expression in PC12 cells that were left untreated (Control) or were treated with PACAP (100 nM, 48 h). The corresponding amplicons were electrophoresed at the end of PCR on 3% agarose gel to assess the amplification of a single DNA band. Statistical analysis was performed using the Student's *t* test. **, $P < 0.01$.

Indeed, we have performed a macroarray validation of the complete set of cDNAs selected from the NIA microarray and the SSH analysis. Quantification of the hybridization signals confirmed the differential expression of numerous clones between control and PACAP-treated cells (Fig. 2A and Tables 3 and 4). As a final verification step, the effect of PACAP on the expression of eight selected genes that represent a range of fold changes was studied individually by using Northern blot and Q-RT-PCR (Fig. 2, B and C). In this anal-

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TABLE 3. Genes up-regulated by PACAP in PC12 cells

Gene	Ratio	Source	Acc.	Unigene
Proliferation/DNA synthesis				
Immediate early response 3	2.4 ± 0.6	NIA	C87164	Mm.25613
ESTs, Highly similar to S21976 RNA-directed DNA polymerase	1.8 ± 0.1	SSH	AI044304	Rn.79185
Growth arrest specific 1	1.7 ± 0.3	SSH/NIA	NM_008086	Rn.41383
Minichromosome maintenance deficient 3-associated protein	1.7 ± 0.3	SSH	AJ006590	Mm.30096
B-cell translocation gene 2	1.6 ± 0.0	NIA	C67946	Mm.903
Transcription factors and regulators				
LIM only 1	2.3 ± 0.1	NIA	AU015284	Mm.12507
Sex comb on midleg-like 1	2.1 ± 0.1	NIA	C86855	Mm.16718
Inhibitor of DNA binding 3	1.8 ± 0.0	NIA	AW557873	Mm.110
MAD homolog 1	1.8 ± 0.6	SSH	U86478	Rn.10635
Transforming growth factor beta 1 induced transcript 4	1.7 ± 0.1	NIA	AU023245	Mm.20927
RNA processing				
ESTs, Highly similar to S50082 nuclear cap binding protein	1.7 ± 0.2	SSH	BI284418	Rn.13482
DEAQ RNA-dependent ATPase	1.6 ± 0.1	SSH	AF316278	Mm.140332
Protein processing/apoptosis				
Serine protease inhibitor	2.7 ± 0.9	IMAGE	AA901070	Rn.128
Peroxiredoxin 5	2.2 ± 0.0	IMAGE	AA859664	Rn.42
Thioredoxin reductase	1.8 ± 0.1	SSH	U63923	Rn.9474
Low density lipoprot. receptor-related prot. associated prot. 1	1.7 ± 0.0	SSH	Z11995	Rn.10293
Bcl2-associated athanogene 3	1.6 ± 0.1	NIA	C79004	Mm.28373
Signaling				
Stromal cell derived factor receptor 2	2.6 ± 0.8	NIA	C86591	Mm.18910
Bone morphogenetic protein 6	1.9 ± 0.1	NIA	C76305	Mm.214548
Delta-like 3	1.8 ± 0.6	IMAGE	AA955549	Rn.23105
Mitogen-activated protein kinase 8 interacting protein 3	1.8 ± 0.0	NIA	AW557032	Mm.43081
ESTs, Highly similar to Faciogenital dysplasia protein homolog1	1.7 ± 0.2	SSH	AW918560	Rn.15417
Aplysia ras-related homolog B	1.7 ± 0.6	NIA	AW536176	Mm.687
Eph receptor A2	1.6 ± 0.0	NIA	AW545284	Mm.2581
S-100-related protein	1.6 ± 0.3	SSH	J03627	Rn.4083
Cytoskeleton				
Thymosin, beta 10	2.6 ± 0.7	SSH/NIA	AA409573	Mm.3532
Keratin complex 2, basic, gene 8	1.8 ± 0.3	NIA	AW544332	Mm.6800
Calponin 3, acidic	1.7 ± 0.2	SSH/NIA	NM_019359	Rn.871
Actinin, alpha 1	1.6 ± 0.1	NIA	AW544340	Mm.23961
Cell matrix/adhesion				
Attractin	2.5 ± 0.6	SSH	NP_112641	Rn.53846
Melanoma cell adhesion molecule	2.4 ± 0.2	NIA	AW555994	Mm.39103
Laminin receptor 1	2.3 ± 0.1	IMAGE	AI044452	Rn.999
Embigin	1.7 ± 0.2	NIA	AW536238	Mm.89123
Ion pumps/transporters				
P450 (cytochrome) oxidoreductase	2.8 ± 0.8	SSH	P00388	Rn.11359
Vesicule/protein trafficking				
Chromogranin B	3.0 ± 0.8	SSH	NM_012626	Rn.11090
Secretory carrier membrane protein 3	2.4 ± 0.2	IMAGE	AA818461	Rn.9151
Rabin 3	1.9 ± 0.3	SSH	U19181	Rn.31889
Golgi SNAP receptor complex member 1	1.7 ± 0.0	SSH	NM_053564	Rn.6390
Synaptosomal-associated protein (Snap25)	1.6 ± 0.2	SSH	AF245227	Rn.24412
Metabolism				
Cytochrome P450, subf. XIB, polypept. 2 (aldosterone synthase)	2.6 ± 0.5	IMAGE	AA924224	Rn.9999
Ornithine decarboxylase antizyme inhibitor	2.2 ± 0.2	SSH/NIA	AU016852	Mm.6775
BetaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3	2.1 ± 0.1	NIA	AW555479	Mm.6826
Methylacyl-CoA racemase alpha	1.9 ± 0.2	IMAGE	AA818115	Rn.2590
NAD(P) dependent steroid dehydrogenase-like	1.9 ± 0.1	SSH	BC019945	Mm.36792
Polypeptide N-acetylgalactosaminyltransferase 7	1.7 ± 0.2	SSH	BC007484	Rn.48729
Glutamate-cysteine ligase, modifier subunit	1.6 ± 0.2	SSH	NM_017305	Rn.2460
Phosphodiesterase 10A	1.6 ± 0.1	SSH	AB027156	Rn.44869
Miscellaneous				
Ring finger protein 34	3.0 ± 0.1	SSH	NM_030564	Mm.7780
ESTs, Highly similar to hypoth. prot. DKFZp434C1415.1	1.5 ± 0.2	SSH	AW435023	Rn.100
Unknown				
ESTs	2.8 ± 1.0	IMAGE	AA818148	Rn.29893
ESTs	2.4 ± 0.1	NIA	AW537092	Mm.28026
ESTs	2.2 ± 0.3	SSH	BC030921	Rn.21415
ESTs	2.0 ± 0.1	SSH	AA900613	Rn.4202
ESTs, Weakly similar to I53063 testic. tumor overexpressed prot.	2.0 ± 0.1	NIA	AW539460	Mm.45132
ESTs	2.0 ± 0.7	SSH	AI071887	Rn.22015
ESTs	1.9 ± 0.3	SSH	BF563306	Rn.47731
ESTs	1.8 ± 0.1	IMAGE	AA964930	Rn.6510
ESTs	1.8 ± 0.1	IMAGE	AA858726	Rn.16888
ESTs, Moderately similar to DJA1_MOUSE DnaJ homolog	1.8 ± 0.2	SSH	NM_021422	Rn.44879
EST	1.8 ± 0.3	NIA	BM250174	
RIKEN clone:4921528E07, Adult male testis cDNA	1.7 ± 0.0	NIA	AU016511	Mm.24760
ESTs, similar to endothelial cell-selective adhesion molecule	1.7 ± 0.0	IMAGE	AI030022	Rn.17089
ESTs	1.7 ± 0.0	SSH	BF548584	Rn.73777
ESTs	1.7 ± 0.5	NIA	AW558883	Mm.182886
ESTs	1.6 ± 0.1	IMAGE	AA964322	Rn.25109
ESTs	1.6 ± 0.2	SSH	BF563306	Rn.47731
ESTs	1.6 ± 0.4	SSH	BG672299	Rn.72610
ESTs, Weakly similar to Prostatic Acid Phosphatase (E.C.3.1.3.2	1.6 ± 0.2	SSH	BM386224	Rn.8329
ESTs	1.6 ± 0.1	IMAGE	AA964885	Rn.11938
ESTs, Moderately similar to hypoth. prot. FLJ23251	1.6 ± 0.2	SSH	BQ205220	Rn.18210
ESTs	1.6 ± 0.1	SSH	AA900613	Rn.26979
ESTs	1.6 ± 0.1	IMAGE	AA956522	Rn.36792
EST	1.5 ± 0.1	IMAGE	AA926253	Rn.15694
ESTs	1.5 ± 0.0	IMAGE	AA924751	Rn.15382
ESTs, Weakly similar to RNA-binding protein 10	1.5 ± 0.2	SSH	BI282892	Rn.17033

The "Source" column indicates the technique used to identify each gene (NIA, microarray containing cDNAs from the NIA 15k mouse embryonic library; SSH, suppression subtractive hybridization; IMAGE, microarray containing rat cDNAs from the IMAGE collection). The ratios and the range variations were determined from two different experiments. Two additional hybridizations were performed on a homemade macroarray to confirm gene expression changes of SSH and NIA clones. Acc., GenBank accession no.

ysis, clones originally identified from mouse and rat microarrays as well as from SSH screening were examined using specific probes and oligonucleotides. Northern blot analysis confirmed that PACAP up-regulates the expression of the genes encoding the transcription modulator Id3, the cell cycle regulator Gas1, the vesicular protein CgB, and the cell adhesion molecule Mcam, and down-regulates the expression of those encoding Mest and Basp1 with unknown function in PC12 cells (Fig. 2B). Using Q-RT-PCR, we confirmed that PACAP significantly stimulates the expression of the transcription factor Madh1 and inhibits that of the signaling protein Ptprr in PC12 cells (Fig. 2C). It appears therefore that the regulation by PACAP of the genes characterized in this study, using microarray/SSH and subsequent macroarray analyses, can be confirmed by independent techniques. These results show that the data that are compiled in Tables 3 and 4 corresponding to up- and down-regulated genes by PACAP in PC12 cells, respectively, are reliable.

We identified 75 genes whose expression was increased and 70 genes whose expression was decreased in PC12 cells after a 48-h exposure to PACAP. A large majority of the genes identified have not been previously described as PACAP-responsive. Of these, 53 were identified from the mouse microarray, 59 were identified from the rat microarray, and 37 were isolated by SSH. Surprisingly, only four of these genes were commonly identified by more than one technique (Tables 3 and 4). This finding could be attributable to several reasons. First, the genes contained in the two types of microarrays used originate from two quite different sources. The NIA microarray is developmentally oriented because the cDNAs printed were derived from a mouse embryonic library, and we were more successful with this microarray to isolate PACAP-regulated genes involved in proliferation, transcription, and cell signaling. The IMAGE (Invitrogen) microarrays contain random clones, mostly expressed sequence tags (ESTs) that represent more than 80% of the cDNAs printed on these membranes. In fact, most of the PACAP-regulated genes identified from the IMAGE microarrays fall in the unknown category in Tables 3 and 4. These rat ESTs represent 3′-noncoding sequences that are probably more difficult to annotate and to match with other genomes including the mouse genome from which the NIA microarray is derived. The characterization of these ESTs will undoubtedly benefit from the advancement of genomic in-

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TABLE 4. Genes down-regulated by PACAP in PC12 cells

Gene	Ratio	Source	Acc.	Unigene
Proliferation/DNA synthesis				
Mini chromosome maintenance deficient 2	0.67 ± 0.10	NIA	AW553939	Mm.16711
Thymopoietin	0.65 ± 0.08	NIA	AW550700	Mm.124
H2A histone family, member Z	0.61 ± 0.01	NIA	AW536811	Mm.916
Cyclin A2	0.57 ± 0.12	NIA	AU020259	Mm.4189
DNA methyltransferase 3A	0.55 ± 0.14	NIA	AW549977	Mm.5001
RGC-32 protein	0.49 ± 0.10	IMAGE	AA858736	Rn.3504
Transcription factors and regulators				
Embryonic lethal, abnormal vision-like 1 (Hu antigen R)	0.62 ± 0.06	NIA	C80193	Mm.119162
High mobility group nucleosomal binding domain 2	0.53 ± 0.00	NIA	AW537812	Mm.911
Transcription factor 4	0.53 ± 0.04	IMAGE	AA956941	Rn.23354
High mobility group box 2	0.48 ± 0.07	NIA	AW546306	Mm.1693
GATA-binding protein 3	0.47 ± 0.02	NIA	C81309	Mm.606
RNA processing				
ESTs, Highly similar to polymyositis/scleroderma autoantigen 2	0.66 ± 0.00	IMAGE	AA955778	Rn.1877
ESTs, Highly similar to 60S ribosomal protein L7A	0.48 ± 0.17	IMAGE	AA900657	Rn.4192
Protein processing/apoptosis				
TCF3 (E2A) fusion partner (Amida)	0.66 ± 0.02	IMAGE	AA997953	Rn.12922
Dorsal protein 1	0.61 ± 0.07	IMAGE	AA924058	Rn.9964
Proteasome (prosome, macropain) subunit, beta type, 7	0.54 ± 0.16	IMAGE	AA955256	Rn.3846
Signaling				
Transforming growth factor, beta 2	0.55 ± 0.06	NIA	C86748	Mm.18213
GTP-binding protein gamma subunit	0.52 ± 0.03	IMAGE	AA899129	Rn.11233
Protein tyrosine phosphatase, receptor type, R	0.49 ± 0.15	IMAGE	AI072547	Rn.6277
Cytoskeleton				
Kinesin-like 5	0.66 ± 0.07	NIA	AU022593	Mm.28386
Cofilin 1, non-muscle	0.62 ± 0.07	IMAGE	AA964009	Rn.11675
Adducin 3 (gamma)	0.59 ± 0.20	NIA	AW549619	Mm.44106
Beta-tubulin T beta15	0.59 ± 0.03	IMAGE	AA899219	Rn.37849
Cell matrix/adhesion				
Lutheran blood group (Auberger b antigen included)	0.62 ± 0.03	NIA	AW553617	Mm.29236
Col3a1 Procollagen, type III, alpha 1	0.58 ± 0.11	NIA	AW550625	Mm.147387
ESTs, Highly similar to CLAUDIN-18	0.58 ± 0.04	IMAGE	AA901239	Rn.4324
Glypican 3	0.43 ± 0.02	IMAGE	AI045921	Rn.9717
Secreted protein, acidic, cysteine-rich (osteonectin)	0.31 ± 0.03	NIA	AW547245	Mm.35439
Ion pumps/transporters				
Potassium intermed./small conduct. ca-activat.channel, subf. N, 4	0.34 ± 0.06	NIA	C86468	Mm.9911
Metabolism				
Thymidine kinase 1	0.64 ± 0.07	NIA	AW544533	Mm.2661
Metabolism (continued)				
Androsterone UDP-glucuronosyltransferase	0.63 ± 0.07	IMAGE	AA858893	Rn.2521
Isocitrate dehydrogenase 2 (NADP+)	0.61 ± 0.05	NIA	AU022195	Mm.2966
Acetyl-Co A acetyltransferase 1	0.58 ± 0.01	IMAGE	AA926170	Rn.4054
Miscellaneous				
Reduced expression 3	0.64 ± 0.05	NIA	AW536404	Mm.14768
Quaking	0.61 ± 0.06	NIA	AU023418	Mm.2655
ESTs, Highly similar to T48344 hypoth. prot. DKFZp434I1614.1	0.59 ± 0.07	IMAGE	AA964657	Rn.11875
Serologically defined colon cancer antigen 33	0.58 ± 0.16	NIA	C88310	Mm.102136
ESTs, Highly similar to T48390 hypoth. prot. DKFZp434C1920.1	0.55 ± 0.07	IMAGE	AA998843	Rn.7108
Brain abundant, membrane attached signal protein 1	0.55 ± 0.01	NIA	AW545587	Mm.29566
ESTs, Highly similar to hypoth. protein DKFZp762K1914.1	0.54 ± 0.11	IMAGE	AA925260	Rn.23118
Alpha-1-acid glycoprotein	0.51 ± 0.11	IMAGE	AI029162	Rn.10295
Mesoderm specific transcript	0.38 ± 0.01	NIA	AW553763	Mm.1089
Unknown				
RIKEN cDNA 1110005F07	0.66 ± 0.02	NIA	AW538517	Mm.21187
ESTs	0.66 ± 0.03	IMAGE	AA997538	Rn.12736
ESTs	0.66 ± 0.00	IMAGE	AA866250	Rn.3017
ESTs	0.66 ± 0.01	IMAGE	AA963844	Rn.8041
RIKEN cDNA 2410008H17 gene	0.64 ± 0.09	NIA	AW536465	Mm.22583
ESTs	0.64 ± 0.00	IMAGE	AA996455	Rn.23608
ESTs	0.64 ± 0.03	IMAGE	AI030241	Rn.18728
ESTs	0.64 ± 0.04	IMAGE	AA858946	Rn.16928
ESTs	0.64 ± 0.01	IMAGE	AI030210	Rn.13113
EST	0.63 ± 0.07	IMAGE	AA956441	Rn.8984
ESTs	0.62 ± 0.04	IMAGE	AA962942	Rn.13986
RIKEN cDNA 5730407I04 gene	0.62 ± 0.11	NIA	AU023009	Mm.102136
ESTs	0.62 ± 0.09	IMAGE	AA997463	Rn.11557
ESTs	0.62 ± 0.03	IMAGE	AA964876	Rn.11842
ESTs	0.62 ± 0.05	IMAGE	AA963838	Rn.11713
ESTs	0.61 ± 0.00	IMAGE	AA925167	Rn.8672
ESTs	0.60 ± 0.02	IMAGE	AA996857	Rn.12436
ESTs	0.59 ± 0.01	IMAGE	AA818132	Rn.21871
ESTs	0.55 ± 0.04	IMAGE	AA964895	Rn.33007
ESTs	0.55 ± 0.01	IMAGE	AI043804	Rn.21687
ESTs	0.54 ± 0.02	IMAGE	AI111919	Rn.8613
ESTs	0.54 ± 0.18	IMAGE	AI029088	Rn.18142
ESTs, Weakly similar to CREB-BINDING PROTEIN	0.54 ± 0.06	IMAGE	AA996888	Rn.12447
ESTs	0.53 ± 0.01	IMAGE	AA900756	Rn.24087
ESTs, Weakly similar to LEG9 RAT GALECTIN-9	0.53 ± 0.06	IMAGE	AI031038	Rn.19533
ESTs	0.51 ± 0.32	NIA	AW547407	Mm.182614
ESTs, Weakly similar to OXYB_oxysterol-binding protein	0.51 ± 0.01	IMAGE	AA901835	Rn.15167
EST	0.46 ± 0.06	IMAGE	AA956227	Rn.32829

For details, see Table 3.

formation. Nevertheless, these latter arrays were used as a complementary material to the NIA microarray to identify additional genes regulated by PACAP. Second, the technologies used for these two kinds of arrays (glass *vs.* nylon, fluorescence *vs.* radioactivity, *etc.*) have quite different sensitivities, and therefore common genes regulated by PACAP should be expressed in the cells at a sufficient level to be detected by both methodologies. Third, the NIA microarray is made of mouse clones, some of which are probably not recognized by targets from rat PC12 cells.

SSH is a different technique that allows a very partial view of the transcriptome compared with microarrays. This approach is interesting, although time-consuming, in that it permits the direct analysis of the transcriptome of the cell model studied and thus the identification of regulated genes that may not be present on defined arrays. Analysis of the 37 genes identified by SSH showed that about half of these (23 genes) are present in the NIA and/or IMAGE (Invitrogen) microarrays used in the present study. Among the latter genes, only four were also found to be changed on the microarrays. These are the genes described above as those that were found changed by more than one technique. This observation implies that microarray analysis probably failed to identify all PACAP-regulated genes, at least in a reproducible manner, although the genes were present on the microarray. For instance, CgB and Madh1, which could not be identified as PACAP-regulated using the NIA or IMAGE microarrays where they are present, respectively, were found changed by SSH, and their variation was confirmed by Northern blot or Q-RT-PCR (Fig. 2). Overall, our results underscore the complementarity of these different techniques and the necessity to use various approaches to study global gene expression changes.

PACAP regulates genes controlling cell growth and differentiation

Among the genes found differentially expressed by microarray or SSH analyses in the presence of PACAP, approximately 40% were unnamed genes, and 55% were genes with a known function that can be classified in various categories (Tables 3 and 4). In PC12 cells, PACAP modified the expression of several genes that are known to be implicated in the regulation of cell growth during development or tumorigenesis in various cell types (Table 3 and 4). PACAP is likely to induce PC12 cell growth arrest by inhibiting the expression of cell cycle regulators, including an MCM protein, the cyclin A2, and thymopoietin, as well as transcription effectors such as high-mobility group and GATA proteins. Interestingly, the levels of these mRNAs were higher in PC12 cells than in nonproliferating chromaffin cells (Table 1). In addition, three of the four genes up-regulated by PACAP and associated

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with proliferation (Table 3), namely immediate early response 3, Gas1, and B-cell translocation gene 2, are direct targets of the tumor suppressor p53 (42–44), indicating that PACAP-regulated pathways may be directly involved in the mechanisms of tumorigenesis.

PACAP may influence PC12 cell differentiation by modulating the biosynthesis of signaling factors that are known to control development of a wide variety of tissues. Indeed, we found that PACAP inhibits TGFβ2 and increases bone morphogenetic protein 6 mRNA levels, two members of the TGFβ family of growth factors that exert pleiotropic effects in nearly all organs, including many roles in neurogenesis (45). Of note is the up-regulation by PACAP of the transcription factors TGFβ1-induced transcript 4 (also known as TSC-22) and Madh1 (also known as Smad1), which are important targets of TGF family members. Collectively, these observations indicate that PACAP may recruit the TGF family signaling pathways to induce cell differentiation and homeostasis. It has been shown that PACAP inhibits TGFβ1-induced apoptosis in a human pituitary adenoma cell line (46), further supporting the notion that PACAP can modulate the effects of this family of growth factors. It is interesting to note that a recent study that analyzed the short-term effects of PACAP (6 h of treatment) on the transcriptome of PC12 cells has revealed the regulation of various early signaling factors that are probably required to initiate differentiation (47).

The effect of PACAP on PC12 cell differentiation is characterized by the sprouting of neuritic extensions. Three proteins up-regulated by PACAP in these cells, ephrin A2, S-100-related protein, and a serine protease inhibitor (Table 3), may be implicated in neurite outgrowth. The expression of the calcium binding S-100 protein is also induced by nerve growth factor in PC12 cells, and transfection of its cDNA has been shown to be able to promote process formation in these cells (48). The receptor tyrosine kinase ephrin A2 is involved in axon guidance and cell migration during embryonic development (49), and the overexpression of this protein is associated with malignancy of some tumors (50). Finally, the effect of serine protease inhibitors on neurite outgrowth has been previously reported in neuroendocrine cells (51). PACAP also regulated the expression of several cytoskeleton proteins that are important effectors of cell morphology remodeling. Most of the PACAP-regulated cytoskeleton proteins were actin-binding proteins that are associated with either polymerization, *e.g.* calponin (52), or depolymerization, *e.g.* cofilin (53) and thymosin β 10 (54), of the actin network. Actin-based motility is critical for both cell migration and extension of neurites (55). In this respect, it is interesting to note that PACAP also stimulated the expression of ras-related homolog (rhoB), a GTPase that regulates actin dynamics to drive neurite extension (55).

PACAP regulates genes controlling cell adhesion

PACAP affected the expression of several genes implicated in cell adhesion and cell-cell contact, which are often altered in tumors. PACAP increased the mRNA levels of laminin receptor 1, which has been shown to be highly expressed in colon carcinoma tissue and lung cancer cells compared with the nontumoral cell counterparts (56, 57). A correlation between the up-regulation of this receptor and the invasive and metastatic phenotype of cancer cells has also been reported (58). The expression of numerous transmembrane glycoproteins including Mcam, attractin, and embigin was increased by PACAP. The expression of Mcam correlates with tumor thickness and metastatic potential of human melanoma cells in nude mice (59). Embigin was found to be more highly expressed in PC12 cells than in adrenochromaffin cells (Table 1). Concurrently, PACAP down-regulated the expression of the extracellular matrix proteins glypican 3 and SPARC, which have been recently associated with tumor progression (60, 61). PACAP also decreased the mRNA levels of an ortholog of the mouse claudin 18, a protein of tight junctions (62), implying that the neuropeptide may inhibit cell-cell contacts in differentiating PC12 cells. Altogether, these data show that PACAP controls the expression of genes that play important roles in cell adhesion and motility, suggesting that the neuropeptide may influence these events in physiological and pathophysiological conditions.

PACAP regulates genes controlling cell survival

In accordance with the known antiapoptotic effect of PACAP in different cell types (6, 63), the present study revealed that the neuropeptide regulates several proteins involved in cell death or survival. Thus, PACAP increased the expression of factors that inhibit protein degradation, such as Bcl2-associated athanogene 3 and the low-density lipoprotein receptor-related protein associated protein 1, as well as detoxifying factors that participate in protection against oxidative stress, *e.g.* peroxiredoxin 5 and thioredoxin reductase (Table 3). It is noteworthy that the expression of antioxidant proteins was higher in adrenomedullary cells than in PC12 cells (Table 2), suggesting that increased expression of these genes is part of the mechanisms underlying the maintenance and survival of differentiated cells, a process in which PACAP may play a physiological function. Moreover, PACAP down-regulated the expression of a novel apoptosis-inducing protein, named Amida, which has been shown to modulate cell death in the brain (64), as well as a component of the proteasome, the subunit β type 7. PACAP also decreased the expression of a calcium-activated potassium channel (Table 4), which could play an important role in cell shrinkage associated with loss of ions that accompanies apoptosis in many cell types (65).

Different kinetics of the effect of PACAP on PC12 cell gene expression

In the present study, we hypothesized that treatment of PC12 cells for 48 h by PACAP would allow the uncovering of early as well as late effects of the neuropeptide during cell differentiation. To assess the validity of this hypothesis, we have examined *post hoc* the kinetics of the action of PACAP in PC12 cells on different genes with various functions (Fig. 3). We found that PACAP rapidly increases (approximately 5-fold at 6 h of treatment) the gene expression of Gas1, a protein involved in growth arrest, and Id3, a transcription regulator (Fig. 3, A and B). The induction of Gas1 was reduced by about 2-fold after 12 h, whereas that of Id3 re-

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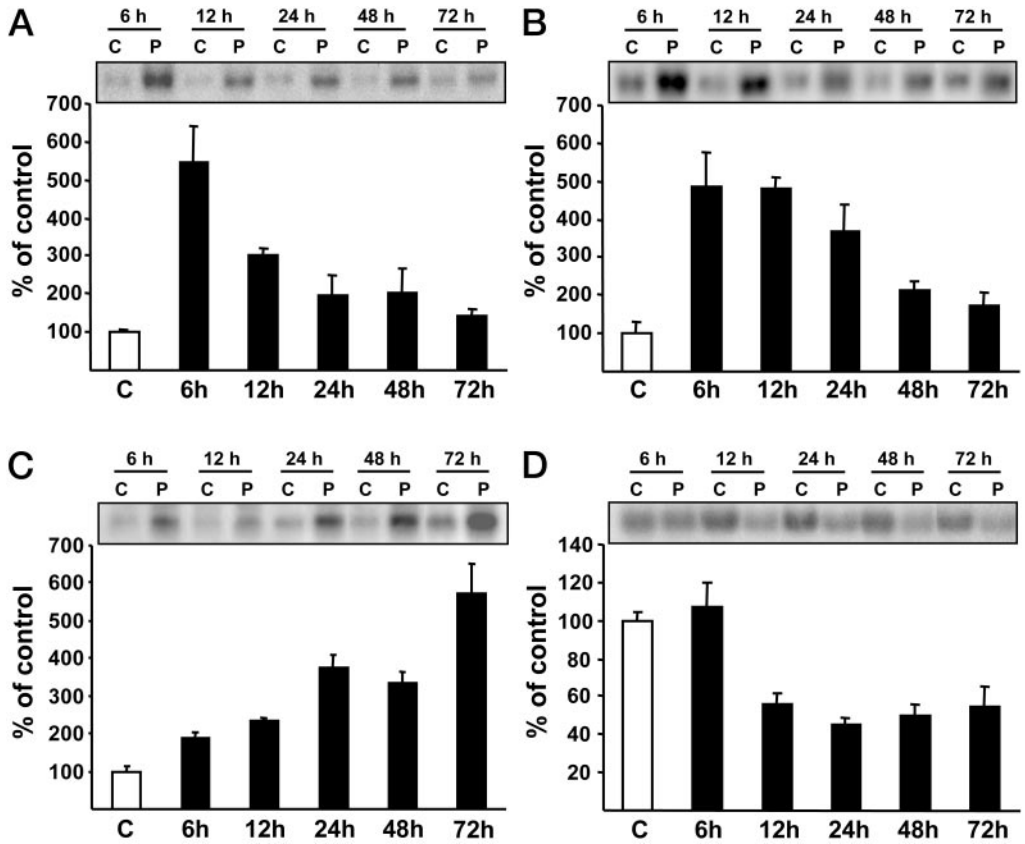

FIG. 3. Kinetics of the effects of PACAP on the expression of four representative genes in PC12 cells. Northern blot analysis of 20 μg of total RNA extracted from control cells or cells treated with 100 nM PACAP for the indicated times was performed to assess the mRNA levels of Id3 (A), Gas1 (B), CgB (C), and Basp1 (D). Data are the mean ± SEM of at least three determinations and are expressed relative to control values.

mained at approximately the same level up to 24 h. These data show that PACAP rapidly induces the expression of genes implicated in cell growth arrest, in line with the results of the study of Vaudry *et al.* (47). Both Gas1 and Id3 genes were still elevated at 48 h, thus confirming our hypothesis, although we may have missed other early genes that had probably returned to baseline within 48 h. The effect of PACAP on the gene encoding the secretory granule protein CgB exhibited a different pattern because the mRNA levels of this protein gradually increased to reach 6-fold at 72 h of PACAP treatment (Fig. 3C). The expression of Basp1, a protein with no known function, also displayed a distinct profile of regulation on PACAP treatment because its mRNA levels were unchanged at 6 h, decreased only at 12 h, and remained inhibited up to 72 h under PACAP exposure (Fig. 3D). Altogether, these data show that PACAP likely modifies the expression of various genes with different kinetics to achieve the gene expression program necessary for cell differentiation.

Comparison of the genes regulated by PACAP with those differentially expressed between PC12 and adrenochromaffin cells

To uncover potential genes whose expression may represent targets of PACAP during sympathoadrenal differentiation, we performed a clustering of genes regulated by the neuropeptide, as identified on the mouse 15K NIA microarray, with those that are differentially expressed between PC12 and fully differentiated adrenochromaffin cells identified on the same array (Table 5). A large proportion of clustered genes belongs to the proliferation and DNA synthesis category, suggesting that the corresponding proteins could represent physiological targets for growth arrest inductive molecules such as PACAP in proliferating sympathoadrenal progenitors. Likewise, the clustering of the transcription regulators Id3 and a LIM only homolog, as well as proteins associated with apoptosis such as the calcium-activated potassium channel and Bcl2-associated athanogene 3, indicates that these factors may also play a role in the differentiation and survival of sympathoadrenal cells. The other clustered genes were mainly those encoding cytoskeleton and cell matrix/adhesion molecules, which are also important effectors of cell differentiation. This comparison shows that several genes regulated by PACAP are also differentially expressed between undifferentiated tumoral chromaffin cells and differentiated adrenomedullary cells, thus arguing for a role of the neuropeptide in the control of cell differentiation in the sympathoadrenal lineage.

In conclusion, we have made use of two types of microarrays representing a total of 25,000 genes and performed a direct differential screening of PC12 mRNAs by SSH to provide insights into the molecular events orchestrated by PACAP to regulate neuronal and neuroendocrine cell differentiation. The majority of the genes regulated by PACAP

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TABLE 5. Genes regulated by PACAP in PC12 cells, and differentially expressed between PC12 and adrenomedullary cells

Gene	Ratio 1	Ratio 2	Acc.	Unigene
Proliferation/DNA synthesis				
B-cell translocation gene 2	1.6	2.6	C87946	Mm.903
Mini chromosome maintenance deficient 2	0.67	3.7	AW553939	Mm.16711
Thymopoietin	0.65	3.1	AW550700	Mm.124
H2A histone family, member Z	0.61	3.2	AW536811	Mm.916
Cyclin A2	0.57	7.3	AU020259	Mm.4189
DNA methyltransferase 3A	0.55	3.0	AW549977	Mm.5001
Transcription factors and regulators				
Lmo1 gene	2.3	2.3	AU015284	Mm.12607
Inhibitor of DNA binding 3	1.8	2.1	AW557673	Mm.110
High mobility group nucleosomal binding domain 2	0.53	2.1	AW537612	Mm.911
High mobility group box 2	0.48	10.0	AW546306	Mm.1693
Protein processing/apoptosis				
Bcl2-associated athanogene 3	1.6	2.2	C79004	Mm.28373
Cytoskeleton				
Keratin complex 2, basic, gene 8	1.8	12.4	AW542449	Mm.6800
Kinesin-like 5	0.66	4.2	AU022593	Mm.28386
Cell matrix/adhesion				
Embigin	1.7	2.6	AW536236	Mm.89123
Lutheran blood group (Auberger b antigen included)	0.62	2.3	AW553617	Mm.29236
Col3a1 Procollagen, type III, alpha 1	0.58	0.39	AW550825	Mm.147387
Ion pumps/transporters				
Potassium interm./small conduct. ca-activated channel, subf. N, 4	0.34	4.2	C86468	Mm.9911
Metabolism				
Ornithine decarboxylase antizyme inhibitor	2.2	3.2	AU016852	Mm.6775
Thymidine kinase 1	0.64	2.7	AW544533	Mm.2661
Miscellaneous				
Reduced expression 3	0.64	4.4	AW536404	Mm.14768
Quaking	0.61	3.8	AU023418	Mm.2655
Serologically defined colon cancer antigen 33	0.58	2.9	C88310	Mm.102136
Brain abundant, membrane attached signal protein 1	0.55	8.1	AW545587	Mm.29586
Mesoderm specific transcript	0.38	15.3	AW553763	Mm.1089
Unknown				
ESTs, Weakly similar to I53063 testic. tumor overexpressed prot.	1.96	2.6	AW539460	Mm.45132

Ratios 1 and 2 were obtained by comparing gene expression in PACAP-treated *vs.* untreated PC12 cells, and PC12 *vs.* adrenomedullary cells, respectively. The fold change limits considered were 1.5 for ratio 1, and 2.0 for ratio 2.

in PC12 cells are involved in cell growth, survival, and adhesion, and many of them have been associated with the progression of various tumors. Moreover, several PACAP-regulated genes are differentially expressed between PC12 cells and adrenomedullary chromaffin cells, further supporting the implication of the encoded proteins in the differentiation events that occur in tumoral and nontumoral cells of this lineage. Additional studies are required to demonstrate the occurrence of these mRNAs and the changes in their expression in sympathoadrenal progenitors as well as in human pheochromocytomas or other neuroendocrine tumors. The present study has also revealed the regulation by PACAP of numerous unnamed genes whose characterization will undoubtedly provide novel information on the effects of this important neuropeptide in the nervous and endocrine systems.

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