A Human cDNA Corresponding to a Gene Overexpressed during Cell Proliferation Encodes a Product Sharing Homology with Amoebic and Bacterial Proteins*

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Marie-Thérèse Prosperi, Didier Ferbus, Isabelle Kareczinski, and Gérard Goubitz†
From the Laboratoire d'Oncogenèse, Institut Curie, 98 rue d'Ulm, 75005 Paris, France

A clone, designated pag, was isolated by differential screening of cDNA libraries made from the untransformed and ras-transformed human mammary epithelial cell line HBL100. This cDNA corresponds to a gene constitutively expressed in most human cells which is induced to higher levels upon serum stimulation in untransformed and ras-transformed HBL100 cells. However, the abundance of the pag transcript is approximately 3-fold higher in transformed as compared to untransformed cells after 7-15 h of serum stimulation. In the promyelocytic leukemia cell line HL60 induced to differentiate the level of pag mRNA starts to decrease between 48 and 72 h following induction. During this period, which represents the commitment phase of differentiation, HL60 cells cease to proliferate. Therefore, in HBL100 and HL60 cells, higher levels of pag gene expression are correlated with cell proliferation. The pag cDNA codes for a 22-kDa protein, devoid of known consensus motifs, and shares 66% homology with a murine gene product (MER5) that is preferentially expressed in erythroleukemia cells during the early period of cell differentiation. In addition, the pag gene product shares approximately 50% identity with a 28-kDa surface antigen of Entamoeba histolytica and a 43-kDa antigen of Helicobacter pylori. Distant relationship was also found with other prokaryotic proteins. The pag cDNA hybridizes to multiple sequences within human and other mammalian genomes and to fewer sequences in chicken and Saccharomyces cerevisiae. Although a true relationship between eukaryotic and prokaryotic genes is difficult to establish, the conservation of pag gene sequences throughout Eukaryota rather suggests that the pag locus belongs to a new class of genes encoding highly conserved proteins.

The three ras oncogenes, Ha, Ki, N-ras, encode highly related 21-kDa proteins (p21) which are ubiquitously expressed and involved in the control of cellular proliferation and differentiation (1). It has been found that 10-20% of human tumors have a mutation in one of these genes leading to the production of p21mut oncoproteins, which are thought to play an important role in the transformed phenotype (2).

The ras oncogenes produce many changes in cellular gene expression by turning on the expression of proteins involved in the regulation of transcription (3, 4). This leads to the aberrant expression of a set of genes, which in turn induces metabolic, ultrastructural, and growth disorders of normal cell life. One approach to identifying genes whose deregulated expression could play a role in transformation is to use the differential cloning technique of cDNA libraries made from transformed and untransformed cells. This technique has been successfully used to identify deregulated genes in avian and rodent transformed cells (5-7), as well as in human tumors (8-10). However, this approach has been only rarely used to identify genes whose expression is altered following ras transformation (11, 12).

In an attempt to isolate abnormally expressed genes in ras-transformed human cells, we have introduced a mutant version of the human Ha-ras gene into the nontumorigenic mammary epithelial cell line HBL100. We succeeded in achieving both morphological transformation and tumorigenic conversion (13). The untransformed and ras-transformed cell lines were used to isolate cDNAs corresponding to genes manifesting an increased expression in ras-transformed cells. We previously used this technique to demonstrate that the α member of the 89-kDa heat-shock protein family is constitutively overexpressed in HBL100 ras-transformed cells (13).

Here, we report the characterization of a novel gene, pag (proliferation associated gene), identified by differential hybridization. Expression of pag and hsp89α genes differ. Although they are both expressed ubiquitously and reach higher levels following serum stimulation in untransformed cells, the pag gene is not constitutively overexpressed in ras-transformed cells. We observed a transient overexpression in ras-transformed HBL100 cells compared to untransformed cells only when cells were induced to proliferate upon serum stimulation. On the other hand, decreased expression was found in HL60 cells in the course of differentiation, suggesting that higher levels of expression are associated with cell proliferation. The pag polypeptide does not contain any known consensus motif and shares approximately 50% identity with parasite and bacterial proteins of unknown function. The possibility that the pag gene could belong to a new family of genes, highly conserved between Eukaryota and Prokaryota, is discussed.

MATERIALS AND METHODS

Cell Culture Conditions—For differential cloning experiments, approximately 3 × 10⁵ untransformed and ras-transformed cells were plated in 100-mm Petri dish and grown for 3 days at 37°C in minimum essential medium supplemented by nonessential amino acids and 10% newborn calf serum. Fresh medium was added overnight before RNA extraction. Serum-starved cells were obtained by

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† To whom correspondence should be addressed. Tel.: 33-1-40-51-66-12; Fax: 33-1-43-26-80-87.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number X67261.
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Molecular Cloning and Nucleotide Sequence of pag cDNA—A cDNA library, primed with oligo(dT), was prepared from polyadenylated mRNA extracted from ras-transformed HBL100 cells. Fifty thousand primary recombinants were screened in duplicate by differential hybridization using 32P-labeled first strand cDNA probes from untransformed and ras-transformed HBL100 cells. In order to detect overexpressed clones resulting only from transformation and not from cell culture conditions, ras-transformed, and untransformed G418-resistant HBL100 cells were grown in parallel. Cultures medium was replaced 16 h before RNA extraction. Clones displaying a stronger hybridization signal with probes prepared from transformed cells were subjected to two further rounds of plaque purification. One of these carrying a new sequence, hybridized with a single mRNA species of 1.2 kb in human cells and showed a differential signal when used to probe Northern blots containing mRNA from untransformed and ras-transformed HBL100 actively growing cells (data not shown). The size of the cDNA insert was shown to be around 900 bp. DNA sequence analysis confirmed the insert size (920 bp) and revealed a stretch of at least 80 adenine, indicating that this clone was probably complete at its 3' end. Since the 920-bp cDNA could be incomplete at its 5' end, due to rearrangement or recombination during the construction of the library, the 920-bp fragment was used to screen a randomly primed cDNA library prepared from ras-transformed HBL100 cells. Three clones extending 17 nucleotides further at the 5' end were isolated. A potential full-length cDNA clone was sequenced. No mismatches were found when compared with the previously determined sequence. Thus, the 917-bp sequence presented in Fig. 1 is likely to represent the entire pag cDNA sequence. Molecular cloning and sequence analysis of the pag gene exons and gene promoter has confirmed this assumption.2

The sequence of the pag cDNA carries a single open reading frame. Postulating the first ATG codon as an initiating codon, the pag sequence consists of a 5'-untranslated region of 60 nucleotides, followed by 597 nucleotides encoding a presumed 199-amino acid polypeptide (Fig. 1). The 3'-untranslated region of 280 nucleotides is composed of 55% A+T base pairs and has an atypical polyadenylation signal, ATTAAA located 11 nucleotides upstream from the polyadenylation site. This hexamer has been shown to serve as an alternative signal for the addition of poly(A) tail (18).

Pag cDNA Encodes a 22-kDa Protein Expressed in HBL100 Cells—The deduced amino acid sequence of pag cDNA is shown in Fig. 1. The calculated molecular weight of the polypeptide is about 22,000. The estimated pl is 8.4. Since the pag cDNA was isolated solely by its mRNA overexpression, known peptide motifs that could suggest the cellular location or the function of the pag protein were searched in the PROSITE data bank (EMBL, release 8.1). No recognizable motif has been found.

To ensure that the deduced sequence indeed encodes a protein of 22 kDa, we raised a polyclonal antiserum, designated PAGA1, against a peptide located in the C-terminal region of pag (position 186–197). To establish the specificity of the PAGA1 antibody for the pag gene product, a DNA fragment containing amino acids 27–199 was fused downstream of the male gene in the pMAL-c2 vector, then introduced and expressed in E. coli. A major protein band of 60 kDa, corresponding approximately to 41 kDa of the maltose-binding protein and 19 kDa encoded by the truncated pag cDNA, was detected by Western blot analysis in samples

1 The abbreviations used are: MeSO, dimethyl sulfoxide; MBP, maltose-binding protein; PAG, polycrylamide gel electrophoresis; bp, base pair(s); kb, kilobase; kbp, kilobase pair(s); MEL, murine erythroleukemia; MERS, murine gene product.

2 M.-T. Prospéri, F. Apiou, B. Dutrillaux, and G. Goubin, manuscript in preparation.
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FIG. 1. The cDNA and predicted protein sequence of pag. Numbers at the left refer to the first nucleotide on the line, and numbers at the right refer to the last amino acid on the line. The putative initiation methionine is shown in capital letters and the polyadenylation signal is underlined.

recovered after affinity chromatography of crude bacterial extracts (Fig. 2A, lane 1). In contrast, the hybrid protein was weakly detectable when immunodetection was performed in presence of competing immunogenic peptide (Fig. 2A, lane 2). Thus, the PAGA1 antiserum indeed recognized the epitope of the pag protein chosen for immunization. Western blot analysis of HBL100 cells, using crude rabbit pag antiserum, revealed multiple protein bands (Fig. 2B, lane 1). Immunodetection performed in presence of competing immunogenic peptide failed to detect two proteins of 22 and 105 kDa (Fig. 2B, lane 2). Because the 1.2-kb messenger RNA is too short to encode a 105-kDa protein, the 22-kDa protein was likely to represent the protein product of the pag gene.

Expression of pag in Various Human Tissues—Since the pag cDNA was cloned from a human mammary cell line, we wished to determine whether its expression was restricted to this cell type. Poly(A) RNA isolated from seven human organs, including heart, brain, placenta, lung, liver, skeletal muscle, and kidney, was analyzed by Northern blot hybridization (Fig. 3). A single 1.2-kb transcript was detected in all organs. The filter was washed and reprobed with β-actin to confirm RNA integrity (Fig. 3). The β-actin expression varies between different organs, thus hampering a direct quantitative comparison with respective pag transcripts. However, if we assume that equal amounts of RNA were indeed loaded onto the gel, pag mRNA levels seemed to be higher in organs having a higher level of proliferation such as kidney, placenta and lung as compared to organs having a low level of proliferation (brain, heart, skeletal muscle, and liver).

Induction of pag Expression by Serum in HBL100 Cells—The association between proliferation and enhanced pag expression, suggested by Northern blot analysis of human organs, was first examined in serum-stimulated untransformed and ras-transformed HBL100 cells. For this purpose, untransformed and ras-transformed cells were serum-starved for 3 days then fed with culture medium containing 10% calf serum. In these culture conditions, growth-arrested HBL100 cells were induced to proliferate. The level of pag gene-specific transcripts was estimated by Northern blot analysis of total mRNA. In serum-starved HBL100 or ras-transformed HBL100 cells, pag mRNA could be easily detected by overnight autoradiography, and its level of expression did not show any significant variation during at least 6 h (Fig. 4). This level was likely to represent the constitutive level of pag mRNA expression in serum-starved HBL100 cells. As already observed for some constitutively expressed genes, such as hsp genes (13, 19), the level of expression increased after serum stimulation. Enhanced expression was seen after 3 h and was
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maximal within 7–15 h following serum addition (Fig. 4). Expression fell off thereafter to reach background levels by 60 h. Although the pag gene was induced by serum in both nontransformed HBL100 or ras-transformed HBL100 cells, its accumulation was approximately 3-fold higher in ras-transformed cells (Fig. 4). This property was not peculiar to the HBL100 cell line used in this assay. Transient overexpression, following serum stimulation of growth arrested HBL100 cells indicate that higher levels of pag gene expression are associated with cell proliferation.

Proteins Related to the pag Gene Product—The deduced amino acid sequence of pag was compared to known protein sequences in the National Biomedical Research Foundation (release 33) and SWISSPROT (release 29) data banks using the FASTA program of Pearson and Lipman (28). Significant homology was found with the MER5 gene product. The cDNA of MER5 codes for a protein of 257 amino acids of unknown function. It was cloned from RNA preferentially synthesized in murine erythroleukemia (MEL) cells during the early period of cell differentiation induced by Me₂SO (29). The homology lies from position 12 in pag and position 70 in MER5 to the C-terminal region. In this region the identity is 66% and rises to 83% when chemically related amino acids are considered (Table I). Only two gaps, one between position 79 and 80 in the MER5 protein and one at position 197 in the pag protein, were introduced during the alignment. Thus, if we exclude the 69 first amino acids of the N-terminal domain of MER5, the pag protein is closely related to MER5.

In addition to the similarity observed with the MER5 gene product, the pag protein revealed significant similarities with amoebic and bacterial proteins. Most closely related to pag is a 29-kDa antigen of pathogenic Entamoeba histolytica, located on the surface of trophozoites (30). The identity was 57% and rose to 73% when chemically related amino acids were considered (Table I). The 26-kDa antigen of Helicobacter pylori (31), a pathogenic bacteria of the stomach, exhibits 48% identity with pag protein and rose to 64%, when conserved amino acid substitutions were considered (Table I). An unpublished sequence of Clostridium pasteurianum, present in the protein data bases shares approximately 50% identity with pag. Distant relationship was also found with an uncharacterized open reading frame (NADH dehydrogenase) located upstream of the coding region of the NADH dehydrogenase of Bacillus subtilis (32), and with a subunit (C22) of the alkyl hydroperoxide reductase of Salmonella typhimurium (33) (Table I).

Fig. 6 shows the amino acid alignment of pag, MER5, and four other proteins bearing significant relationship with pag. Several positions along the sequences are perfectly conserved. The highest degree of similarity lies between position 41 and 56 of the pag protein and has the following consensus sequence: Phe(Leu)-Phe-Phe-Tyr-Pro-Leu(X)-Asp-Phe-Thr-Phe-Val-Cys-Pro-Thr-Glu-Ile(Leu). No known motif was

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**FIG. 2.** Immunodetection of purified MBP-pag fusion protein and pag protein in HBL100 cells. MBP-pag fusion protein (3 μg) purified by affinity chromatography from a bacterial lysate obtained from MBP-pag expressing E. coli and crude extract of HBL100 cells (10 μg) were separated through SDS-10% polyacrylamide gel and transferred to nitrocellulose membranes using standard immunoblotting techniques (16). A, immunoblot of purified MBP-pag fusion protein probed with the PAGA1 antibody in the absence (lane 1) or presence (lane 2) of competing immunogenic peptide (30 μg/ml). B, immunoblot of crude extracts of HBL100 cells probed with the PAGA1 antibody in the absence (lane 1) or presence (lane 2) of competing immunogenic peptide (30 μg/ml).

**FIG. 3.** pag expression in normal human tissues. A human multiple tissue Northern blot containing equal amounts of poly(A) RNA (2 μg) was purchased from Clontech Laboratories and hybridized using a pag full-length cDNA clone as a probe (top panel). RNA integrity was verified by washing the filter and reprobing it with a β-actin probe (bottom panel), according to the supplier protocol (Kodak X-OMAT AR film; exposure time, 4 h).

were used as control. A slight decline of pag mRNA levels was observed after 48 h and was clearly seen after 72 h to reach very low levels after 80 h (Fig. 5, top left panel). Stepwise decrease of pag mRNA was not observed during the same period in HL60 cells grown in the absence of Me₂SO when corrected for variability in RNA loading (Fig. 5, top right panel). Therefore, decreasing levels of pag mRNA are associated with HL60 cell differentiation and do not result from culture conditions such as nutrient depletion. To ensure that the differentiation process was indeed triggered upon addition of Me₂SO, RNAs were subsequently hybridized with a human c-myc probe. As previously described (21–24), c-myc RNA was highly expressed in HL60 cells actively growing in the absence of Me₂SO (Fig. 5, middle right panel), whereas it was dramatically reduced within few hours of exposure to Me₂SO and remains extremely low thereafter (Fig. 5, middle left panel). Interestingly, the period from 48 to 72 h constitutes what has been termed the commitment phase of differentiation which also corresponds to the period HL60 cells cease to proliferate (20, 24–27). Thus, the levels of pag mRNA and proliferation are decreasing in parallel. These data together with those demonstrating enhanced pag mRNA expression following serum stimulation of growth arrested HBL100 cells indicate that higher levels of pag gene expression are associated with cell proliferation.
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![Graph](image)

**Fig. 4. Time course of the pag gene expression after serum stimulation.** Total cytoplasmic RNA (5 μg) isolated from nonstimulated (−) and serum-stimulated (+) HBL100 cell lines, at the indicated times, was analyzed by Northern blot. (N) is a clone of nontransformed HBL100 cells containing the pSV2neo vector (HBL100/neo1), and (R) a clone of ras-transformed HBL100 cells, designated HBL100/ras1 (13). The pag probe was a full-length cDNA clone. Underexposed autoradiograms (4 h) were scanned and the results used to plot the curve in arbitrary units. The level of pag expression found in serum-starved cultures was considered as the base line and subtracted from the values obtained upon serum stimulation. Each point represents the percentage of the maximum mRNA level found in ras-transformed HBL100 cells 7 h after serum stimulation and arbitrarily defined as 100%. For reproduction purposes, a longer exposure (Kodak X-OMAT S film, 16 h) is presented in the figure.

![Blot](image)

**Fig. 5. Northern blot analysis of the pag message during the course of differentiation of HL60 cells.** Total cellular RNA was extracted from HL60 cells at the indicated times, grown in the presence (left panels) or in the absence (right panels) of 1.3% Me2SO (DMSO), then probed for pag mRNA (top panels). Filters were subsequently hybridized with a XbaI-BgIII fragment as a probe, containing exon 2 of the human c-myc gene (middle panels). Hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served to normalize for the uneven loading of RNAs in different lanes (bottom panels).

found to correspond to this conserved sequence, or to other less conserved sequences among the consensus amino acid sequences of the PROSITE data bank (EMBL, release 8.1).

**Evolutionary Conservation of the pag Gene**—The evolutionary conservation of the pag gene was examined in cellular DNA from different mammals, chickens, and yeasts. Under moderate stringent hybridization (30% formamide, 5 x SSC, 42 °C) and washing (0.1 x SSC, 50 °C) conditions, the pag cDNA hybridized to multiple sequences within human and mammalian genomes and to fewer sequences in chicken genome (Fig. 7). It also hybridized with a 2.2-kbp fragment of yeast Saccharomyces cerevisiae DNA. Additional fragments of 10 and 3.3 kbp were also seen on overexposed autoradiograms (data not shown). Therefore, the pag cDNA represents a highly conserved gene sequence.

**Table 1**

Relationship of pag protein to known protein sequences

| Assay | MER5 | E. histolytica | H. pylori | ndh | C22 |
|-------|------|----------------|-----------|-----|-----|
| Percent identity | 66 | 57 | 48 | 35 | 33 |
| Percent similarity | 17 | 16 | 16 | 22 | 26 |
| Percent identity | 83 | 73 | 64 | 57 | 59 |
| + similarity | Position on pag | 12-197 | 12-194 | 12-194 | 25-189 | 15-151 |

**DISCUSSION**

Activation of a ras gene has a pleiotropic effect on cell growth leading ultimately to a transformed phenotype. We have chosen to compare a ras-transformed human cell with its untransformed counterpart in order to identify genes whose deregulation may participate in transformation. A novel cDNA clone, pag was thus isolated.

The pag cDNA encodes a 199-amino acid polypeptide with a predicted molecular mass of 22 kDa, assuming that initiation of translation starts at the first ATG codon. Using an antibody raised against a peptide located within the C-terminal part of pag, Western blot analysis revealed a 22-kDa protein expressed in HBL100 cells. This suggests that the predicted structure of the pag polypeptide is correct. However, we cannot rule out that an another codon is used for initiation of translation. For example posttranslational modifications of a smaller polypeptide could lead to a product with an apparent molecular mass of 22 kDa. Direct sequencing of the purified pag protein will identify the initiation codon. In the absence of such data, we can, however, conclude that the polypeptide encoded by the open reading frame is expressed...
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in HBL100 cells. The size predicted from the cDNA sequence is similar to that estimated by Western blot analysis.

The pag gene was found to be transcribed in most human tissues suggesting that a constitutive level of expression is necessary for cell life. Following serum stimulation of growth-arrested HBL100 cells, pag expression dramatically increased above the constitutive level. On the other hand, pag mRNA expression decreased during differentiation of HL60 cells, in particular when they ceased to divide (48-72 h), whereas it remained high in actively growing HL60 cells. Therefore, in this cell system, variations of the pag expression did not appear to be due to culture conditions such as nutrient depletion or acidification of the medium. Although we ignore its precise function, this gene was designated pag (proliferation associated gene) since higher levels of pag mRNA expression are associated with cell proliferation.

Comparison of the pag amino acid sequence with MER5 revealed a high degree of homology (Table I). The MER5 gene was first identified as a cDNA, cloned from RNA preferentially synthesized in murine erythroleukemia during the early period of cell differentiation (29). Antisense RNA of the MER5 gene inhibits the differentiation of murine erythroleukemia, suggesting that the MER5 gene product is necessary for completing differentiation (34). The homology spans most of the pag protein, and corresponds to region 70-257 of the MER5 protein. The difference in size between the pag and MER5 gene products as well as comparison of their gene promoters (29) indicate that pag is not the human counterpart of the murine MER5 gene. Moreover, pag is expressed in most human tissues whereas MER5 is preferentially expressed in immature erythroblast cells (29). pag mRNA expression decreases during the course of differentiation of HL60 cells, whereas MER5 mRNA expression increases during the early period of differentiation of MEL cells (24-48 h). Although pag and MER5 display a different pattern of expression during differentiation, it is tempting to speculate that pag and MER5 share a common domain involved in the cellular response to either proliferation or differentiation signals. This domain may encompass the whole region of homology or be limited to common motifs. In either case, the absence of homology either proliferation or differentiation signals. This domain may encompass the whole region of homology or be limited to common motifs. In either case, the absence of homology

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identity (57%) with a 29-kDa surface antigen. Homology reached 73% when conserved amino acid substitutions were considered. The 26-kDa antigen of H. pylori was also found closely related to pag. H. pylori is a pathogenic strain of Gram-negative bacteria associated with diseases of the gastrointestinal tract including carcinoma (35, 36). Mild treatment of H. pylori removes the 26-kDa antigen from the cells, suggesting that the protein might be located on the cell surface (31).

Work is in progress to obtain antibodies against the native pag protein, to determine whether pag is associated with the plasma membrane despite the absence of consensus sequences for membrane localization.

We have shown that pag is a serum-inducible gene highly related to eukaryotic and prokaryotic proteins. Two hypotheses can be drawn from these observations. Close analogy of protein sequences between prokaryotes and eukaryotes can reflect the conservation of gene products necessary for cellular survival. Alternatively, this homology may represent a case of convergent evolution of proteins. Since E. histolytica and H. pylori are both pathogenic organisms, one can speculate that antigens sharing structural relationship with a host protein have been selected for their role in the infectivity of human cells. Although a true evolutionary relationship between eu- karyotic and prokaryotic proteins may be difficult to establish, the high homology shared between these proteins, together with the high degree of conservation throughout Eukaryotae, rather favors the hypothesis that the pag gene product belongs to a novel class of highly conserved proteins.

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