Identification of a New Interferon-α-inducible Gene (p27) on Human Chromosome 14q32 and Its Expression in Breast Carcinoma

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ABSTRACT

A new complementary DNA, p27, has been cloned and sequenced from estradiol-treated MCF7 human breast carcinoma cells. It encodes a putative highly hydrophobic protein of 122 amino acids which has a 33% overall sequence similarity to the product of the 6-16 gene (R. L. Friedman, S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark, Cell, 38: 745–755, 1984), which is transcriptionally induced by interferons of the α/β type. We demonstrate here that the p27 gene, which is located in band q32 of human chromosome 14, is also induced by interferon-α in human cell lines of different origin and that expression is independent of the presence of estradiol receptor in the cells. High levels of p27 RNA were found in vivo in approximately 50% of primary human breast carcinomas (21 were tested by Northern blotting). In situ hybridization to some of the p27-overexpressing tumors showed that the p27 RNA is localized in cancer cells and sometimes also in fibroblastic cells of tumor stroma. p27 RNA levels in the tumors did not correlate with the presence of estrogen receptor or with the expression of the estrogen-induced pS2 gene. Further studies are now necessary to elucidate the cause of p27 gene overexpression in breast carcinoma and in particular to determine whether it corresponds to chromosomal rearrangements in the 14q32 region and/or to induction by interferons of the α/β type.

INTRODUCTION

In the industrialized world, with few exceptions, breast cancer is a leading cause of cancer deaths in women (1, 2). Early detection based on mammography screening has improved the prognosis of the disease (3, 4), but it is clear that new treatments are necessary and that a prerequisite to the development of new therapeutic agents is a better understanding of the disease at the molecular level (2).

Estrogens play an important part in the genesis of breast cancer (5, 6), and about one-third of the tumors require the presence of estrogens for growing (7). In order to understand the molecular basis of estrogen action, several groups have screened for genes which are under estrogen control in the ERα- and/or ERβ-type. We demonstrate here that the p27 gene, which is located in band q32 of human chromosome 14, is also induced by interferon-α in human cell lines of different origin and that expression is independent of the presence of estradiol receptor in the cells. High levels of p27 RNA were found in vivo in approximately 50% of primary human breast carcinomas (21 were tested by Northern blotting). In situ hybridization to some of the p27-overexpressing tumors showed that the p27 RNA is localized in cancer cells and sometimes also in fibroblastic cells of tumor stroma. p27 RNA levels in the tumors did not correlate with the presence of estrogen receptor or with the expression of the estrogen-induced pS2 gene. Further studies are now necessary to elucidate the cause of p27 gene overexpression in breast carcinoma and in particular to determine whether it corresponds to chromosomal rearrangements in the 14q32 region and/or to induction by interferons of the α/β type.

Received 4/28/93; accepted 6/25/93.

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1 Supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Mutuelle Générale de l’Éducation Nationale, the Ministère de la Recherche et de l’Espace (contrat 92H.091), the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer, the Fondation pour la Recherche Médicale Française, the Groupement des Entreprises Françaises pour la lutte contre le Cancer, and the Fondation Jeanjean.

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4 The abbreviations used are: ER, estradiol receptor; IFN, interferon; DMEM, Dulbecco’s modified Eagle’s medium; cDNA, complementary DNA; poly(A)+, polyadenylate.

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tion of the purified recombinant phage DNA with the EcoRI restriction enzyme and subcloned into M13 vector, following standard procedures. Sequencing was performed by the dideoxy-chain termination method, using the Sequenase/ deaza-dGTP Reagent Kit (U. S. Biochemicals, Cleveland, OH). cDNA sequences were analyzed with the DNASTAR software (DNASTAR, Madison, WI), and amino acid sequences were aligned using the method of Needleman and Wunsch (17).

Gene Mapping. p27 gene mapping was carried out by in situ hybridization on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 h. 5-Bromo-oxyuridine (60 µg/ml) was added to the medium for the final 7 h of culture to ensure a posthybridization chromosomal banding of good quality. The p27 cDNA, containing 598 base pairs cloned in the pBSII SK + vector (Stratagene, La Jolla, CA), was titrated labeled in a nick-transcription to a specific activity of 1.5 × 10⁴ dpm/µg. The radiolabeled probe was hybridized to metaphase spreads at a final concentration of 25 ng/ml of hybridization solution, as previously described (18). After the slides were coated with nuclear track emulsion (NTB2; Kodak, Rochester, NY), they were exposed for 19 days at 4°C before development. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa solution, and metaphases were photographed. R-banding was then performed by the fluorochrome-photolysis-Giemsa method, and metaphases were rephotographed before analysis.

RNA in Situ Hybridization. In situ hybridization was performed on formaldehyde-fixed paraffin-embedded tissue sections as previously described (13). In brief, 8-µm-thick rehydrated and acid-treated sections were digested with proteinase K (2 µg/ml-30 min, 37°C), followed by overnight hybridization (0.3 n NaCl-50% formamide, 50°C) with a 32P-labeled p27 antisense RNA probe (specific activity, 10⁸ cpm/µg). The sections were RNase treated (20 µg/ml, 30 min, 37°C) and stringently washed (2× standard sodium citrate, 50% formamide, 60°C, twice for 2 h). p27 antisense RNA was obtained by in vitro transcription of the full-length p27 cDNA from the pBSII SK + vector. Autoradiography was for 3 to 4 weeks using the NTB2 emulsion. After exposure, the slides were developed and stained with hematoxylin. The ER status of tumors was determined by immunohistochemical analysis as previously described (19).

RESULTS

Cloning of the p27 cDNA. A total of 60,000 plaques from a substracted MCF7/estradiol-positive cDNA library was differentially screened using one probe derived from MCF7 cells cultured in the presence of estradiol [MCF7/estradiol-positive] and a second probe derived from MCF7 cells cultured in the absence of estradiol [MCF7/estradiol-negative]. A clearly higher hybridization signal with the MCF7/estradiol-positive than with the MCF7/estradiol-negative probe was given by 318 plaques, and 252 of these "differential clones" corresponded to the estradiol-inducible pS2 gene (9). In a second screening of the remaining clones, 21 showed a differential signal, but 15 corresponded, again, to pS2. After a third screening, 4 clones still showed a differential signal, and their inserts were then used to probe Northern blots prepared with MCF7/estradiol-positive and MCF7/estradiol-negative RNAs. Three of them hybridized to a 0.6-kilobase RNA band which was present at high levels only on the MCF7/estradiol-positive blot (Fig. 1, lanes 1 and 2). The fourth insert did not hybridize to any RNA (data not shown). The 3 cDNAs that hybridized to the 0.6-kilobase estradiol-induced transcripts were sequenced and found to correspond to the same gene that we termed p27 (Fig. 2).

Characterization of the p27 cDNA. p27 RNA is the same size as pS2 RNA (9, 20), and it is also similarly induced by estradiol in the MCF7 cells (Fig. 1, lanes 1 and 2). However, the p27 cDNA sequence indicates that it corresponds to an entirely different gene (Fig. 2) and that it contains an open reading frame which codes for a putative protein of 122 amino acids with a calculated molecular weight of 11,528. The putative initiation codon (nucleotides 55–57) is found in a typical eukaryotic initiation consensus sequence (21), and a single polyadenylation signal is seen 21 nucleotides upstream from the start of a possible poly(A) tail. A hydrophathy plot (not shown) indicates that the predicted p27 protein is highly hydrophobic and, therefore, possibly corresponds to a membrane protein, but it contains no typical signal peptide sequence at the NH₂ terminus.

A database search (National Biomedical Research Foundation-Protein Identification Resource, Washington, DC, 1992, release 32) showed that the p27 protein has highest amino acid similarity (41% identity in a 116 amino acid overlap, 33% overall) to a 130-amino acid protein which has been predicted from the sequence of a cDNA cloned from T98G neuroblastoma cells (22), and termed 6–16 (Fig. 2B). The 6–16 gene is induced by IFN-α/β in a variety of human cells, including T98G neuroblastoma cells and HeLa cells (22, 23). The function of the putative 6–16 protein is unknown, but it is suggested to be a membrane protein, with a highly charged carboxy-terminal tail and a typical signal peptide sequence at the NH₂ terminus, both apparently absent in the putative p27 protein.

Fig. 1. Comparative Northern blot analysis of p27 and pS2 RNAs in human cell lines. Northern blots were prepared with 8 µg total RNA from cells at confluency, in each sample. Filters were successively hybridized with 32P-labeled human cDNAs corresponding to the p27 gene, the pS2 gene [an estradiol-inducible gene (9)], and the 36B4 gene [an ubiquitously expressed gene (19)]. p27 and pS2 transcripts were detected at 0.6 kilobases, and 36B4 transcripts were detected at 1.5 kilobases. Autoradiography was for 16 h.

p27 Gene Localization to Chromosome 14q32. In situ hybridization on human chromosome preparations was carried out using 32P-labeled p27 cDNA. In the 200 metaphase cells examined after in situ hybridization, there were 564 silver grains associated with chromosome 14. The majority of these were found in the q32 band (Fig. 3). Thus, the p27 gene is localized on the 14q32 band of the human genome, which is frequently involved in chromosomal translocations associated with lymphoid malignancies (24, 25).

p27 Gene Expression in Human Cell Lines and Its Induction by Interferon-α. A number of human cell lines, cultured in the presence of estradiol-containing fetal calf serum, were analyzed for p27 gene expression by Northern blotting. In contrast to pS2 RNA, p27 RNA was not detected in the ER(+) BT-474 cells (Fig. 1, lane 3), and very low levels of p27 RNA were seen in the ER(+) T-47D cell line (Fig. 1, lane 4). However, high levels of p27 transcripts were seen in the ER-negative HBL-100 (Fig. 1, lane 5) and SK-BR-3 (Fig. 1, lane 6) cells. The p27 gene was not expressed in the other human epithelial and nonepithelial cell lines which were tested (Fig. 1, lanes 7, 8, and 10–14) with the exception of KATO III (Fig. 1, lane 9), a cell line derived from an ER-negative gastric carcinoma.

Altogether, these observations indicate that p27 gene expression does not correlate with ER expression in human cell lines, although it is induced by estradiol in the MCF7 cells. Since the putative p27 protein exhibited 41% identity with the 6–16 protein, for which the expression is induced by IFN-α/β (22, 23), we tested whether p27
gene expression is induced by IFN-α. IFN-α was indeed found to stimulate p27 gene expression in the 3 cell lines tested, of either epithelial (MCF7 and HeLa cells) or mesenchymal (HFL1 fibroblasts) origin (Fig. 4), which do not constitutively express the gene (the low level of p27 RNA in MCF7 cells before IFN-α addition was most probably due to the presence of residual levels of estradiol in the culture, as indicated by the simultaneous presence of pS2 RNA). p27 RNA was induced in a dose-dependent manner by IFN-α, and the effect was specific for the p27 gene since IFN-α did not increase pS2 or 36B4 gene expression in the same cells (Fig. 4).

**p27 Gene Expression in Breast Carcinoma.** p27 RNA expression was evaluated by Northern blot analysis of 21 primary invasive breast carcinomas, 1 breast cancer bone metastasis, and 3 breast fibroadenomas. High levels of p27 transcripts, by comparison with those observed in the 3 fibroadenomas, were found in approximately 50% of the primary breast carcinomas tested and in the bone metastasis (Fig. 5A and data not shown). Furthermore, p27 gene overexpression was not correlated with ER expression in the tumors (for instance, ER was not immunodetected in carcinoma 5 or 7, but 90% of cancer cells were ER(+) in carcinoma 8; Fig. 5A and data not shown), and the pattern of p27 gene expression differed from that of pS2 (Fig. 5A) which is a known marker of ER-dependent breast carcinomas (19, 26). The p27 gene was also expressed at significant levels in some normal non-breast tissues, including colon, stomach, and lung (Fig. 5B).

Seventeen primary invasive breast carcinomas, 4 breast cancer metastatic lymph nodes, 1 breast cancer bone metastasis, and 4...
Fig. 3. Localization of p27 gene on chromosome 14q32. Top and middle, 2 partial human metaphases showing the specific site of hybridization to chromosome 14. Top, arrowheads, silver grains on Giemsa-stained chromosomes after autoradiography. Middle, chromosomes with silver grains subsequently identified by R-banding (fluorochrome-photolysis-Giemsa technique). Bottom, idiogram of the human G-banded chromosome 14 illustrating the distribution of labeled sites for the p27 cDNA probe.

p27 gene expression was induced in breast fibroadenomas by in situ hybridization for p27 gene expression. p27 transcripts were not detected at significant levels in the normal cells of the tissue sections (Fig. 6, a and b, and data not shown), but a low level of p27 RNA was observed in fibroblasts of 2 fibroadenomas (data not shown). In contrast, high levels of p27 transcripts were detected in cancer cells of 13 of the primary breast carcinomas and 4 of the metastases (one metastatic lymph node was negative) (Fig. 6, a–d, g, and h, and data not shown). In the primary tumors, both invasive (Fig. 6, a and b) and in situ (Fig. 6, c and d) cancer cells expressed the p27 gene. p27 gene expression was also observed in stromal cells of 4 primary tumors and 2 metastases (Fig. 6, e and f, and data not shown). Furthermore, cancer cells that were surrounded by p27-expressing stromal cells did not express the p27 gene (Fig. 6, e and f), but they did in other parts of the same tumor where the gene was not expressed in the stromal cells (Fig. 6, a–d). We found no p27 gene expression in inflammatory cells of the tumor stroma, and the levels of p27 transcripts did not appear to be correlated with the intensity of the inflammatory component of tumors (data not shown).

DISCUSSION

We have identified a new gene, p27, which maps to position 14q32 on the human genome. The p27 cDNA contains an open reading frame which codes for a putative 122-amino acid highly hydrophobic protein, suggesting that it is a membrane protein. The protein sequence shows 33% overall similarity to a previously reported putative protein which is encoded by the 6–16 gene (22, 23). Although expression of the p27 gene is induced by estradiol in MCF7 breast cancer cells, it is not dependent on the presence of ER in other cell lines, nor in primary breast carcinomas where p27 gene expression does not correlate with that of the estrogen-induced pS2 gene. Evidently, high levels of p27 transcripts were detected both in ER-negative cell lines and in ER-negative breast carcinomas. After noting the homology on the protein level between p27 and 6–16, which is highly induced by IFN-α/β in a variety of human cell lines (22, 23), we tested and found that the p27 gene is also induced by IFN-α in cells of different origin.

The IFNs, which can be classified as cytokines, are part of the body's natural defense responses to tumors, and it has been suggested that the IFN-α/β genes represent a class of tumor suppressor genes (27–30). They exert antitumor effects both by affecting the functioning of the immune system and by direct action on certain tumor cells (31). IFNs are believed to mediate these multiple effects by inducing or repressing the synthesis of several proteins (32–34), and the p27 protein may belong to this group. Whether p27 gene expression is controlled by IFNs in vivo is unknown, but it is not unlikely, since most cell types appear capable of producing and binding IFN-α/β (32, 34). Furthermore, p27 RNA was present both in the neoplastic cells and in the fibroblastic cells of breast carcinomas, an observation which is consistent with the in vitro results, showing p27 gene induction by IFN-α in cell lines of both epithelial and mesenchymal origin. That increased p27 gene expression was observed in only some breast tumors may indicate that these tumors produce higher levels of, or have higher sensitivity to, IFN-α/β than others. Alternatively, increased p27 gene expression in breast carcinoma may be a result of chromosomal rearrangements in the 14q32 region. In this respect, we note that the 14q32.1 locus, to which the p27...
Fig. 5. Comparative Northern blot analysis of p27 and pS2 RNAs in human tissues. A, breast tumors: lanes 1–10, invasive carcinomas; lane 11, bone metastasis; lanes 12 and 13, fibroadenomas. B, normal tissues. Northern blot analysis was carried out as described in the legend to Fig. 1.

Fig. 6. In situ hybridization of p27 RNA on breast cancer tissue sections. a, c, e, and g, bright-field photomicrographs of paraffin-embedded tissue sections (×100) stained with hematoxylin; b, d, f, and h, dark-field photomicrographs of the same sections in which p27 transcripts appear as white silver precipitates after in situ hybridization with 35S-labeled p27 antisense RNA. a–f, 3 distinct areas of the same tumor: a and b, infiltrating ductal carcinoma in which p27 transcripts are detected in neoplastic cells (C) but not in stromal cells (S) or in normal epithelial cells (N); c and d, 2 ducts containing ductal in situ carcinomas (*), one of which expresses the p27 gene; e and f, ductal infiltrating carcinoma in which p27 transcripts are detected in stromal cells (S) but not in neoplastic cells (C). g and h, breast cancer metastatic lymph node expressing the p27 gene.

ACKNOWLEDGMENTS
We thank R. Ceredig for critically reading the manuscript, H. V. J. Kolbe for suggestions during the work, I. Stoll, C. Wendling, and M. Loriot for technical assistance, and the Michigan Cancer Foundation for MCF7 breast cancer cells.

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