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Activation of c-Ki-*ras* coexists with c-*myc* amplification in cells from a nude mouse tumor induced by the human breast carcinoma cell line SW 613-S

Guido Carloni*+, Brigitte Champ°, Marie-José Vilarem†, Christian Lavialle* and Roland Cassingena*

*Laboratoire de Génétique Cellulaire, ER 278, CNRS, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France, +Institute of Experimental Medicine, CNR, Rome, Italy, °Institut de Pathologie Moléculaire, UA 1147, CNRS, Paris and †Laboratoire de Génétique Cellulaire et Moléculaire des Leucémies Aigües, U301, INSERM, Hôpital Saint-Louis, Paris, France

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In vitro transfection experiments have shown that cooperation between two different oncogenes can confer a fully malignant phenotype to primary rodent cells. We have previously reported that SW 613-Tul cells, derived from a tumor induced in a nude mouse by the human breast carcinoma cell line SW 613-S, showed a 30-fold amplification of the c-*myc* gene. In the present work, we show that these cells also harbor an activated c-Ki-*ras* gene capable of inducing the formation of foci upon transfection of NIH 3T3 cells with SW 613-Tul genomic DNA. Our results suggest that both the c-*myc* and c-Ki-*ras* oncogenes, activated by two different mechanisms, may cooperate in the full expression of the tumorigenic phenotype of SW 613-Tul cells.

Breast carcinoma; Gene activation; Gene amplification; *ras* gene; *myc* gene

1. INTRODUCTION

Activation of cellular proto-oncogenes by various genetic alterations is believed to play a major role in tumor formation and/or progression [1–3]. In human malignancies, proto-oncogenes have repeatedly been found to be altered by chromosome translocation, gene amplification or specific point mutations [1–3]. The first two mechanisms of activation lead to disruption in the regulation of expression of the gene, whereas the latter results in a functionally altered gene product, such as occurs for genes of the *ras* family [1–3]. Mutations in *ras* genes, first unveiled by transfection assays on NIH 3T3 cells, are now detected with an increased frequency in human tumors, owing to the advent of the more sensitive technique of hybridization with specific oligonucleotide probes [4,5].

In the case of human breast cancer, a highly polymorphic and heterogeneous group of neoplasias [6], several studies have reported the frequent occurrence of c-*myc* or c-*erbB2/neu* gene amplification [7–11]. Transforming genes, as detected by transfection assays, have been described in only a few human mammary tumor cell lines: c-Ha-*ras* in HS578T [12]; c-Ki-*ras* in MDA-MB-231 [13] and H-466B [14]; N-*ras*, *mcf*-2 and *mcf*-3 in MCF-7 [15]. *mcf*-3 turned out to be the c-*ros*1 gene activated during gene transfer [16], whereas *mcf*-2 remains to be characterized.

Here, an activated c-Ki-*ras* gene was identified by transfection of NIH 3T3 cells with DNA from SW 613-Tul, a subline derived from a tumor induced in a nude mouse by the human breast carcinoma cell line SW 613-S [17]. Of interest is the

Correspondence address: G. Carloni, Laboratoire de Génétique Cellulaire, IRSC, BP no.8, 94802 Villejuif Cedex, France

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fact that we have previously shown that the c-*myc* gene is amplified 30-fold in SW 613-Tul cells [17].

2. MATERIALS AND METHODS

2.1. *Cell lines*

The establishment and characterization of the SW 613-Tul cell line have been described [17]. PCC-4 is a mouse teratocarcinoma cell line, in which the c-Ki-*ras* gene is amplified 10–20-fold [18].

2.2. *DNA transfection assay*

Total cellular DNA was prepared according to Gross-Bellard et al. [19]. DNA transfection was carried out by the calcium phosphate coprecipitation method [20], essentially as in [21]. Briefly, 4×10^5 NIH 3T3 cells were seeded per Petri dish in 5 ml Eagle's minimal essential medium (MEM) supplemented with 10% newborn calf serum (Gibco). 1 day later, 20 μg high-M_r DNA coprecipitated with calcium phosphate were applied to each dish for 5 h and the transfection was followed by a glycerol shock [22]. Cultures were maintained in MEM/10% calf serum with medium being changed every 3–4 days. Once the cell monolayers reached confluency, the serum concentration was reduced to 5%. Morphologically transformed foci were scored at 14–18 days. Individual foci were picked using cloning glass cylinders, transferred into 24-well microplates and propagated under low serum selective culture conditions.

2.3. *DNA blotting*

DNA from NIH 3T3 transformants was digested with *Eco*RI, separated by horizontal electrophoresis in agarose gels, transferred to nitrocellulose filters and hybridized with either of two DNA probes radiolabeled by nick-translation: (i) human repetitive DNA sequences prepared by reannealing sonicated and denatured DNA from human cells, as described by Houck et al. [23]; (ii) the pKBE-2 plasmid [24] carrying the v-Ki-*ras* oncogene from Kirsten murine sarcoma virus. Hybridizations were carried out for 24 h under conditions of low stringency (30% formamide, 0.75 M NaCl, 0.075 M Na citrate, 1 × Denhardt solution, 100 μg/ml of sonicated-denatured salmon sperm DNA, at 42°C). In the case of the human repetitive DNA probe, the hybridization mixture was supplemented with 50 μg/ml of denatured NIH 3T3 cell DNA.

2.4. *Immunoblotting of* ras *p21 protein*

Cell lysates were prepared in Laemmli sample buffer supplemented with 5% 2-mercaptoethanol and proteins were separated by electrophoresis on a 15% polyacrylamide gel [25]. Proteins were then transferred by electrophoresis onto an 0.22 μm nitrocellulose filter (Schleicher and Schüll), as described by Towbin et al. [26]. The filters were preincubated for 3–4 h at 37°C in a blocking solution containing 3% (w/v) bovine serum albumin and 1% (w/v) bovine immunoglobulins in TNE buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 2 mM EDTA). Hybridization was carried out for 2 h in TNE buffer containing 0.1% Nonidet P40, using as a probe the rat

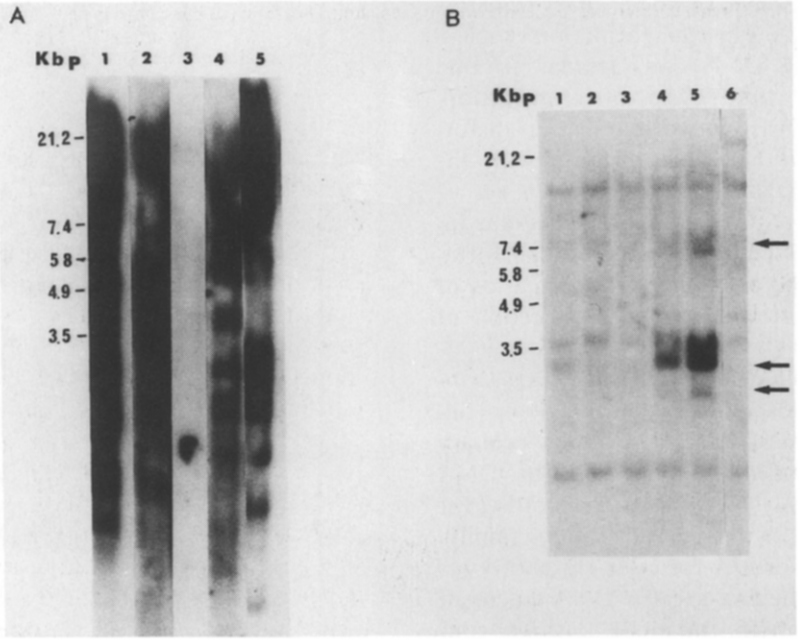

Fig.1. Southern blot hybridization of *Eco*RI-digested DNA from NIH 3T3 transformants obtained after transfection of SW 613-Tul DNA. (A) Hybridization with the human repetitive DNA probe of genomic DNA from two primary transformants (lanes 1,2), NIH 3T3 control cells (lane 3) and two secondary transformants (lanes 4,5). (B) Hybridization with the pKBE-2 probe of DNA from primary transformants (lanes 1–5) and NIH 3T3 cells (lane 6). Lanes 4 and 5 show DNA samples from the same primary transformant at passage 5 and 6, respectively. Arrows indicate the position of the human c-Ki-*ras* gene-specific bands.

monoclonal antibody Y13-259 [27]. After washing, the filters were incubated with a biotinylated anti-rat immunoglobulin antibody, followed by peroxidase-streptavidin (Amersham). The specific protein bands were revealed by reacting the filters with a peroxidase staining solution prepared in 100 ml phosphate-buffered saline by adding 50 mg 4-chloro-1-naphthol predissolved in 1 ml ethanol and 50 μl of 30% H_2O_2.

3. RESULTS

High-M_r DNA from the SW 613-Tul tumor cell line was tested for its transforming activity by transfection of NIH 3T3 cells. Morphologically transformed foci appeared 2 weeks later with an efficiency of 0.055 focus/μg DNA. Each focus was picked, grown into mass culture and the cellular DNA was extracted. The DNA from these primary transformants was first tested by Southern blotting for the presence of human repetitive sequences. Fig.1A (lanes 1,2) demonstrates the presence of a large number of DNA fragments reactive with the human repetitive DNA probe in the DNA of two such transformants, under conditions where no hybridization was detectable with control NIH 3T3 DNA (lane 3). A second cycle of transfection was carried out with the DNA of one primary transformant (fig.1A, lane 1). The transformation efficiency (0.043 focus/μg DNA) was similar to that observed for the first cycle of transfection. Analysis of the DNA from two secondary transformants by Southern blot hybridization with the human repetitive DNA probe showed a set of defined bands (fig.1A, lanes 4,5). These results indicated that SW 613-Tul cells contained transforming sequences capable of inducing the formation of foci in two consecutive cycles of transfection of NIH 3T3 cells.

In order to determine whether these transforming sequences corresponded to any known oncogene, the DNA from several primary transformants induced by SW 613-Tul DNA transfection was analysed by Southern blotting for the presence of one of the genes of the *ras* family. Hybridization with probes for c-Ha-*ras* and N-*ras* did not detect any human-specific DNA fragment (not shown). In contrast, the pKBE-2 probe, containing the v-Ki-*ras* gene [24], detected *Eco*RI fragments of 6.7, 3.0–3.1 and 2.5 kbp, in addition to the endogenous mouse c-Ki-*ras* fragments, in the DNA of two primary transformants (fig.1B, lanes 1 and 4,5, respectively). No hybridization was observed with the DNA of two other primary transformants (fig.1B, lanes 2,3), but these turned out to have lost the transfecting human DNA upon subculturing, when tested with the human repetitive DNA probe. The high intensity of the bands observed with the transformant DNA shown in lanes 4,5 of fig.1B is presumably due to the amplification of the transfected oncogene.

Expression of the p21 *ras* protein in the primary transformants harboring the activated c-Ki-*ras* gene from SW 613-Tul was investigated by immunoblotting using the broadly reactive monoclonal antibody Y13-259 [27]. Fig.2 (lanes b,c) shows that both transformants expressed clearly detectable amounts of p21 protein, although at a slightly lower level than that expressed by the PCC4 mouse teratocarcinoma cell line used as a positive control (lane a) and in which the c-Ki-*ras* gene is amplified at least 10-fold [18]. Comparatively, the p21 *ras* protein band was barely detectable in lysates from untransformed NIH 3T3 cells (not shown).

Our results indicate that transformation of NIH

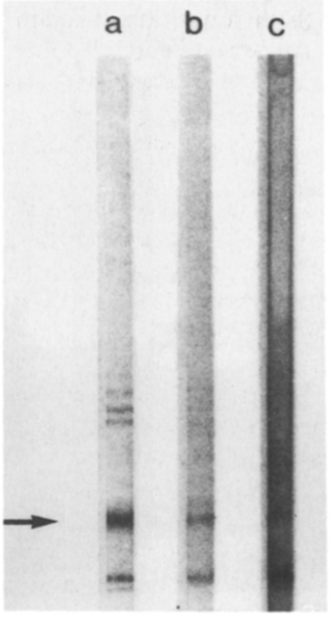

Fig.2. Detection of the p21 *ras* protein by immunoblotting with the monoclonal antibody Y13-259 in lysates from PCC4 mouse teratocarcinoma cells, used as a positive control (lane a) and from two independent primary transformants harboring the activated c-Ki-*ras* gene from SW 613-Tul (lanes b,c). The arrow indicates the position of the p21 *ras* protein band.

3T3 cells by transfection of SW 613-Tul DNA was due to a c-Ki-*ras* oncogene. The fact that independent primary transformants harbored the human c-Ki-*ras* gene indicates that transforming activity was not acquired during the transfection process but that this gene is already activated in the SW 613-Tul cell line used as DNA donor.

4. DISCUSSION

We have shown here that the SW 613-Tul cell line harbors an activated c-Ki-*ras* gene, as tested by transfection assays on NIH 3T3 cells. The transforming activity of this gene is presumably due to a point mutation located at either codon 12, 13 or 61, leading to an amino acid change at one of these positions in the encoded p21 protein. Indeed, all genes of the *ras* family from human tumors active in transfection assays which have been analysed to date have been found to be altered by one of these mutations [3]. Southern blot analysis of *Sac*I digests of primary transformant as well as SW 613-Tul DNA (not shown) did not reveal the new restriction site expected to be present if a guanine to cytosine substitution had occurred at the first position of codon 12 in the activated c-Ki-*ras* gene, as described previously in the case of several other human tumor cell lines [28]. Further work is required to determine by sequence analysis the precise nature and location of the activating mutation.

As mentioned above, the c-*myc* gene is amplified 30-fold in SW 613-Tul [17]. Thus, this cell line harbors two activated oncogenes, c-*myc* and c-Ki-*ras*, which may cooperate in the expression of the tumorigenic phenotype of these cells. SW 613-Tul was derived from a nude mouse tumor induced by the human breast carcinoma cell line SW 613-S [17]. We have recently demonstrated, by analysis of clonal isolates, that SW 613-S is heterogeneous with regard to the amplification level of the c-*myc* gene (Lavialle, C. et al., submitted). Only clones with a c-*myc* amplification level of at least 30-fold are capable of inducing the formation of rapidly growing tumors upon s.c. inoculation of nude mice. Once the activating mutation in the c-Ki-*ras* locus of SW 613-Tul has been identified, it will be of interest to investigate its presence by hybridization with an appropriate oligonucleotide probe in the genome of the parental SW 613-S cell line, as well as in that of the various clones derived therefrom.

The coexistence of two oncogenes activated by different mechanisms in the same tumor cell (e.g. a mutated *ras* gene and a translocated or amplified c-*myc* gene) has been reported in some human hematopoietic malignant cells [29] and in some human solid tumors and tumor-derived cell lines [30,31]. This has led to speculation that consecutive activation of two or more oncogenes may contribute to the multistep process of carcinogenesis. Such a hypothesis is further supported by in vitro transfection experiments showing that two different oncogenes can cooperate in inducing the full malignant transformation of rodent embryo fibroblasts [32,33]. Within this context, the SW 613-S human breast carcinoma cell line and the diverse sublines derived from it, such as SW 613-Tul, constitute an interesting experimental model for investigating the cooperative role of the two activated oncogenes c-Ki-*ras* and c-*myc* in tumor progression.

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