Expression of the onc Gene of the Kirsten Murine Sarcoma Virus in Differentiated Rat Thyroid Epithelial Cell Lines

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(Accepted 2 August 1984)

SUMMARY

A systematic study has been performed using a series of differentiated rat thyroid epithelial cell lines either uninfected or infected with Kirsten murine sarcoma virus (KiMSV), to determine the levels of the p21 product of the v-ras-Ki oncogene in transformed and normal cell lines. The p21 levels have been assayed by SDS-polyacrylamide gel electrophoresis of immunoprecipitates of 35S-labelled cell extracts and by a GDP binding assay. All cell lines analysed showed a significant increase in the levels of p21 after transformation with KiMSV compared to the p21 levels of uninfected and untransformed differentiated thyroid cells. The results reported here confirm the potential ability of the v-ras-Ki oncogene product to transform epithelial cells. They show, furthermore, that not only is p21 present in some epithelial cells transformed by KiMSV, but also that it is functionally active, as has been shown for fibroblasts transformed by the same virus, and that its functioning is maintained after passaging in vivo of the transformed cells.

INTRODUCTION

Kirsten (KiMSV) and Harvey (HaMSV) murine sarcoma viruses owe their transforming ability to two ras genes, named v-ras-Ki and v-ras-Ha, respectively (Ellis et al., 1982). The 21000 mol. wt. proteins encoded by these genes (p21 kis and p21 has) (Shih et al., 1979a), whose nucleotide sequences have been established (Dhar et al., 1982; Tsuchida et al., 1982), are functionally and immunologically related, sharing about two-thirds of their tryptic peptides (Ellis et al., 1981); antibodies directed against p21 has also immunoprecipitate p21 kis (Furth et al., 1982). p21 kis and p21 has are also immunologically related to a cellular p21 found at low levels in normal cells (Langbeheim et al., 1980). Both p21 proteins have a unique affinity for guanine-containing nucleotides, and the binding assay for GDP or GTP has been reported to correlate well with the functionality of the p21 proteins (Scolnick et al., 1979).

The transforming proteins of KiMSV and HaMSV have been extensively studied in fibroblasts transformed by these viruses. Until now, however, the expression of the v-ras-Ki oncogene has not been studied in cultured cell lines of well-defined epithelial origin, expressing markers typical of differentiation.

In our laboratory it has recently been demonstrated that Kirsten sarcoma virus, besides being capable of transforming cells of mesenchymal origin, such as fibroblasts, is also capable of transforming in vitro differentiated (Fusco et al., 1982) and undifferentiated (Fusco et al., 1981) cells of a clear epithelial derivation, such as rat thyroid cells. We have further shown that the differentiated functions of such cells are irreversibly shut off after transformation with KiMSV (Fusco et al., 1982). In rat thyroid cells transformed with a temperature-sensitive mutant of the virus, these functions are not re-acquired after the resumption of the normal state when the cells are shifted to the non-permissive temperature (39 °C) (Colletta et al., 1983).
The aim of the present study was to assay the levels and the activity of the p21 *kis* protein and p21 mRNA in a variety of normal or KiMSV-transformed rat thyroid epithelial cell lines available in our laboratory in order to establish a relationship between p21 expression and phenotypic properties of the cell. The results obtained demonstrate, for the first time, that p21 is expressed in epithelial cells transformed by KiMSV. This protein seems to be responsible for the transformation of differentiated epithelial cells, in the same way as for fibroblasts, since its level is clearly higher in transformed thyroid cell lines compared with uninfected untransformed thyroid cells.

**METHODS**

**Cells and viruses.** Derivation, transformation and maintenance of the thyroid-derived cell lines have been described previously (Ambesi-Impiombato & Coon, 1979; Ambesi-Impiombato et al., 1980; Fusco et al., 1981, 1982; Colletta et al., 1983). In brief, FRT-L and T-79 are two independently obtained epithelial thyroid cell lines, derived from Fischer rats, expressing differentiation markers [i.e. thyroglobulin (TG) synthesis, iodide uptake and thyrotropic hormone (TSH) sensitivity]. The FRT-L line is a homogeneous clonal population, whereas the T-79 cell line consists of a heterogeneous population of pure epithelial cells (Fusco et al., 1982).

T-79 KiKi and FRT-L KiKi are T-79 and FRT-L cells infected and transformed with KiMSV-KiMuLV; FRT-L KiMol and FRT-L KiMol *ts* are FRT-L cells infected and transformed with a wild-type strain and a *ts* mutant (371) of KiMSV-MoMuLV (Shih et al., 1979b). T-79, T-79 KiKi, FRT-L KiKi, FRT-L KiMol and FRT-L KiMol *ts* are uncloned populations. A6 and A18 are cloned populations derived from FRT-L KiMol *ts* (Colletta et al., 1983); T-79-T are cells established, as a continuous line, from a T-79-KiKi induced tumour (Fusco et al., 1982). KiMSV-KiMuLV was obtained from supernatant fluids of KiMSV-KiMuLV-infected NRK 58967 cells (Roy-Burman & Klement, 1975); KiMuLV was obtained from supernatant fluids of KiMuLV-infected NIH 3T3 cells.

**Metabolic labelling of cells and immunoprecipitation.** Procedures for cell labelling, preparation of cell extracts and immunoprecipitation were performed essentially as described by Shih et al. (1979a) and by Furth et al. (1982). Immunoprecipitations were carried out using both rat antisera containing antibodies to p21 *has* (Shih et al., 1979a) and monoclonal antibody Y 13-259 to p21 (Furth et al., 1982). Both antibodies recognize p21 *has* and p21 *kis* proteins (Shih et al., 1979a; Furth et al., 1982). Rat antisera and monoclonal antibodies were generous gifts of Drs T. Shih and M. Furth (NIH, Bethesda, Md., U.S.A.), respectively.

**GDP binding assay.** The GDP binding assay was performed as described by Scolnick et al. (1979). Each reaction mixture contained in 0·3 ml, 20 mM-Tris-HCl pH 7·2, 100 mM-sodium chloride, 5 mM-magnesium chloride, 1% Triton X-100, 0·009 mM-[3H]GDP (sp. act. 10 Ci/mmol), and 100 μg proteins. Ten μl of rat anti-p21 serum or monoclonal antibody Y 13-4 [Furth et al., 1982] were added. The antigen–antibody complexes were precipitated by the addition of either 50 μl of a 10% suspension of *Staphylococcus aureus* or 250 μl of rabbit anti-rat IgG serum.

Monoclonal antibody against thyroglobulin (kindly provided by Dr G. Rossi, Naples, Italy) was used as control.

**Cytoplasmic RNA extraction.** Cytoplasmic RNA was extracted from cultured cells essentially as described previously (Bilello et al., 1980). In brief, cells were harvested, washed three times with cold phosphate-buffered saline and centrifuged in a Sorvall HL-8 rotor at 1500 r.p.m. for 5 min. Cells were then resuspended in cold lysis buffer (140 mM-NaCl, 10 mM-Tris-HCl pH 7·4, 3 mM-MgCl2) at a final concentration of 20 x 10⁶/ml, and NP40 (0.5~) was added. Nuclei were pelleted in a HL-8 rotor at 3000 r.p.m. for 5 min. The supernatant fluid was treated with proteinase K (500 l/xg/ml) in the presence of SDS (1%), and then extracted three times with phenol–chloroform and precipitated with 2 vol. ethanol in 0·3 M-NaCl.

**Dot hybridization.** Nitrocellulose filters were soaked first in water and then in 20 x SSC (1 x SSC: 0·15 M-NaCl, 0·015 M-trisodium citrate) for at least 4 h. RNA resuspended in 10 μl of 6 x SSC was spotted on the filter at the concentrations indicated in the legend to Fig. 3. The filter was dried in a vacuum oven at 80 °C for 2 h, pretreated for 24 h at 42 °C with a prehybridization buffer: 5 x Denhardt's solution, 5 x SSC, 50% formamide, 1% glycine, 250 μg/ml denatured calf thymus DNA and then hybridized in a hybridization buffer: 1 x Denhardt's solution, 5 x SSC, 10% dextran sulphate, 50% formamide, 100 μg/ml calf thymus DNA with a 32P-labelled nick-translated v-ras-Ki-specific probe, the clone HiHi 3 (sp. act. 2 x 10⁸ c.p.m./μg), for 24 h at 42 °C. The HiHi 3 clone was a generous gift of Dr N. Tsuchida (Ellis et al., 1981). This clone is 1·0 kbp long and covers the entire coding region of the p21 protein. After hybridization the filter was washed twice in 0·1 x SSC plus 0·1% SDS at 58 °C for 30 min and then exposed to X-ray film with the use of intensifying screens at -80 °C.

**RESULTS**

Different epithelial cell lines derived from rat thyroid gland have been transformed in our laboratory by three strains of the Kirsten sarcoma virus (KiMSV-KiMuLV, KiMSV-MoMuLV, *ts* 371 KiMSV) (Fusco et al., 1981, 1982; Colletta et al., 1983). Two of these, the cloned FRT-L and the uncloned T-79 cell line, maintain thyroid gland-specific differentiated functions: they
Fig. 1. SDS–gel electrophoresis of 35S-labelled extracts from uninfected and KiMSV-transformed cells immunoprecipitated with rat anti-p21 serum, monoclonal antibody, Y 13-259, to p21 or preimmune serum. Cells were labelled overnight in methionine-free medium containing 2% dialysed foetal calf serum and 200 μCi/ml [35S]methionine (> 600 Ci/mmol, Amersham). Each immunoprecipitation sample, containing 8 x 10^6 TCA-precipitable c.p.m., was incubated with 5 μl of serum or monoclonal antibody and run on 12% polyacrylamide gel. (a) Lanes 1 and 3, FRT-L KiKi and FRT-L KiMol, respectively, immunoprecipitated with anti-p21 serum; lanes 2 and 4, FRT-L KiKi and FRT-L KiMol immunoprecipitated with preimmune serum. (b) Lanes 1 and 3, FRT-L KiMol and uninfected FRT-L, respectively, immunoprecipitated with Y 13-259; lanes 2 and 4, FRT-L KiMol and uninfected FRT-L immunoprecipitated with a control monoclonal antibody (anti-rat thyroglobulin). (c) Lanes 1 and 3, T-79 KiKi and T-79-T, respectively, immunoprecipitated with Y 13-259; lanes 2 and 4, T-79 KiKi and T-79-T, respectively, immunoprecipitated with a control monoclonal antibody (anti-rat thyroglobulin).
synthesize and secrete thyroglobulin (TG), accumulate iodide and are dependent on thyrotropic hormone (TSH) for growth. These properties are lost after transformation (Fusco et al., 1982), the cells change in growth pattern and morphological properties, and become malignant.

Since the transformed state is dependent on the acquisition of the Kirsten virus genome, and the v-ras-Ki gene in particular, we have studied the expression of this gene in our epithelial cells, immunoprecipitating [35S]methionine-labelled cellular extracts with anti-p21 antibodies. Rat anti-p21 has serum specifically immunoprecipitated two proteins with a mol. wt. of about 21000 in both FRT-L KiKi and FRT-L KiMol cells (Fig. 1a, lanes 1 and 3, respectively). These two proteins are almost completely absent in lanes 2 and 4 of Fig. 1(a), where the same amount of 35S-labelled cellular extract was immunoprecipitated with a preimmune rat serum.

Monoclonal antibodies that recognize p21 kis were then used to reduce the high background. Fig. 1(b) shows immunoprecipitates, obtained with monoclonal antibody Y 13-259, of normal FRT-L cells and FRT-L KiMol cells. p21 was clearly immunoprecipitated by specific antibodies in FRT-L KiMol cells (Fig. 1b, lane 1), whereas it is not visible in untransformed FRT-L cells (Fig. 1b, lane 3). Nevertheless, in other experiments performed by us, a faint double band is present in untransformed FRT-L cells (data not shown). This is in agreement with published data (Furth et al., 1982; Langbeheim et al., 1980; Chang et al., 1982; Der et al., 1982; Tabin et al., 1982), and indicated that a c-ras-coded p21 is present at basal levels in the normal cells of many species. p21 is specifically immunoprecipitated also in the T-79 cell line transformed by KiMSV-KiMuLV, as shown in Fig. 1(c), lane 1. It is interesting to notice that amounts of p21 similar to those present in the transformed T-79 cells are present in the T-79-T cell line (Fig. 1c, lane 3). This cell line was obtained from a tumour caused by injection of T-79 KiKi into a syngeneic rat (Fusco et al., 1982). These cells show in vitro all the features of being transformed: alteration in morphology as well as clonability in agar. These cells, however, do not release virus (A. Fusco et al., unpublished data). In all these experiments, p21 was present in multiple forms, with molecular weights ranging from 21000 to 17000 (Langbeheim et al., 1980; Tabin et al., 1982).

The function of p21 in fibroblasts has been related to its capacity to bind guanine-containing nucleotides (Scolnick et al., 1979). It was important, therefore, to verify whether p21 was also functionally active in these rat thyroid epithelial cell lines. In Fig. 2, where a GDP-binding assay is represented, it is demonstrated that the presence of p21 in the epithelial cell lines is closely related to functionality. In fact, the cell lines that express the highest levels of p21 (FRT-L KiKi, FRT-L KiMol and T-79-T), as indicated by gel electrophoresis of immunoprecipitates, show the greatest ability to bind GDP, whereas the FRT-L cell line, which shows very low levels of p21, has low or no GDP-binding activity. The FRT-L cell line has recently been infected with the ts mutant 371 of the KiMSV (Colletta et al., 1983). The FRT-L KiMol ts cells behave as transformed cells only at the permissive temperature (33 °C), whereas they display a normal phenotype when shifted to the non-permissive temperature (39 °C). We were unable to detect the presence of p21 or GDP-binding activity either in the ts transformed cell line nor in several clones obtained from the parental cell line (data not shown). It appears likely that neither monoclonal antibodies nor anti-p21 rat serum were able to recognize the mutated p21 protein under the conditions employed. To overcome this problem, we analysed total RNA extracted from the ts cell lines for the presence of RNA sequences homologous to the HiHi probe used by us. It has been shown that the coding region for KiMSV p21 is located close to the 5' end of the viral RNA and the complete genomic RNA is more efficient in specifying synthesis in vitro of p21 than sub-genomic poly(A)-containing fragments (Shih et al., 1979a). Therefore, all the RNA sequences detected by our hybridization procedure represent mRNA sequences coding for the p21 protein. By this analysis we have shown that mRNA coding for p21 is indeed present in quite large amounts in all of the KiMSV-transformed cells, when compared to normal uninfected cells or to FRT-L cells infected with the leukaemogenic helper alone (KiMuLV) (see Fig. 3). As expected, mRNA is present at both permissive and non-permissive temperatures, in agreement with the observation that the ts mutation is connected with the thermolability of p21 protein (Shih et al., 1979b).
DISCUSSION

Acute oncogenic retroviruses possess a set of genes that are part of the viral genome and that can induce tumours of mesenchymal origin in vivo and transform fibroblasts or haematopoietic cells in vitro. Recently, several studies have linked one family of viral oncogenes, the ras family, to which v-ras-Ha and v-ras-Ki belong (Ellis et al., 1981), with the sequences present in several human tumour DNAs (Slamon et al., 1984), which are able to transform fibroblasts in vitro by transfection. Unfortunately, very little is known about the function of the proteins encoded by these genes in the normal regulation of the cell life cycle and about the mechanisms by which these proteins induce the cell to behave as malignant.

For this reason, we have focused our attention on the ability of Kirsten sarcoma virus to transform in vitro cultured cells of a well-defined epithelial origin, such as rat thyroid epithelial differentiated cells (Fusco et al., 1982). In the present study we have correlated the presence and the activity of the p21 transforming protein of KiMSV, with the transformed phenotype in the various thyroid epithelial cell types transformed by KiMSV. Furthermore, we have analysed the presence of p21 in all types of cells before and after transformation with KiMSV, in order to correlate p21 expression with the expression of phenotypic markers of thyroid differentiation.

The transformed cell lines studied are derived from two different thyroid epithelial cell lines, FRT-L and T-79, expressing three typical markers of thyroid differentiation (thyroglobulin production, iodide uptake and TSH dependence for growth). We have also studied whether there is a change in the expression of the v-ras-Ki oncogene after passaging in vivo of the virus-transformed cells. Finally, we have measured the function of p21 in these cell lines by the GDP-binding assay. This activity has been correlated with transformation since the GDP-binding capacity of p21 is thermolabile in ts transformed cells (Scolnick et al., 1979).

Our cellular system is suitable for studying the role of p21 in the change of expression of the differentiated phenotype. We have demonstrated that transformation by KiMSV blocks the typical differentiated functions of the thyroid gland, since neither TG nor TG-specific mRNA
are synthesized, nor is iodide concentrated by the cells after transformation (Fusco et al., 1982). In this paper we demonstrate that these transformed lines produce very high levels of functionally active p21 protein as compared to the non-transformed cells. These high p21 levels are possibly responsible for the acquisition of the transformed state (tumourigenicity, growth in agar, etc.) and also for the block of differentiated functions. However, p21 expression does not seem to be continuously required in order to cause this block in the differentiated properties of the transformed cell lines. Such a block, in fact, is not reversible when cells infected with the ts mutant of KiMSV are shifted to the temperature non-permissive for transformation (Colletta et al., 1983). It is noteworthy that significant changes in the levels and activity of p21 after transformation with KiMSV have been observed together with changes in both morphology and growth rate of all transformed cell lines. These data correlate well with our previous work on the FRT-L cell line infected by is mutant 371 of KiMSV. In this system some properties, such as morphology and growth rate, appear to be under strict control of p21, since they drastically change when the cells are shifted from the permissive to the non-permissive temperature, where p21 is probably inactive, and vice versa (Colletta et al., 1983).

Our previously published results (Fusco et al., 1981, 1982; Colletta et al., 1983), together with those presented here, provide a clear demonstration that one of the genes of the ras family of genes encoding p21 is responsible for in vitro transformation of cells of epithelial origin. The results obtained in recent years using human tumours of epithelial origin and the transfection assay on NIH 3T3 cells are in line with our studies of transformation in vitro of thyroid epithelial cells. Most of the transforming genes present in human carcinomas are related to the ras family and it has been reported that this family of oncogenes seems to be preferentially activated in tumours of epithelial origin (Pulciani et al., 1982; Slamon et al., 1984). This relationship between histological derivation and transforming genes suggests that transformation of specific types of tissues is caused by a specific type of oncogene or oncogene family.

In the light of the above-mentioned observations and of the results obtained by us with these transformed thyroid cell lines, it should be interesting to verify the role of the ras gene family, or of their normal counterpart, in normal development and in human and experimental neoplasms of the thyroid gland.

This work was supported by the Progetto Finalizzato Controllo della Crescita Neoplastica of the Consiglio Nazionale delle Ricerche (C.N.R.) contratto no. 82.00431.96 and no. 83.00974.96.

REFERENCES


*(Received 28 February 1984)*