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Expression of the c-*src* Protooncogene in Human Skin Tumors[1]

Angelika Barnekow,[2] Eberhard Paul, and Manfred Schartl

Institut für Medizinische Virologie, Frankfurter Strasse 107 [A. B] and Zentrum für Dermatologie, Gaffkystrasse 14 [E. P.], Fachbereich Humanmedizin der Justus-Liebig-Universität Giessen, D-6300 Giessen, and Max-Planck-Institut für Biochemie, Genzentrum, D-8033 Martinsried [M. S.], Federal Republic of Germany

ABSTRACT

Retroviral oncogenes are genetic elements, the expression of which is responsible for the transformed phenotype of cells. These genes are derived from normal cellular DNA sequences called cellular protooncogenes, which are present in all human cells and seem to have potential transforming ability in tumors of nonviral origin, since it is possible that they undergo structural alterations and/or changes in their expression.

Human skin tumors were analyzed in this study with respect to the expression of the c-*src* protooncogene, the cellular homologue of the Rous sarcoma virus transforming gene, by measuring the enzymatic activity of its gene product, the pp60$^{c\text{-}src}$ kinase activity. Tyrosine-specific kinase activity was detected in all skin tumors tested. The expression pattern of the c-*src* gene product in the melanomas tested was differential and varying kinase levels in different metastases from the same patient were detected. The elevation of kinase activity as compared to normal skin ranged from about 4- to 20-fold.

INTRODUCTION

The genomes of all vertebrates, including humans, contain a variety of sequences which are potentially oncogenic. Some of these highly conserved sequences are homologous to the transforming genes of certain RNA tumor viruses and were thus designated cellular protooncogenes (1–5). To date, three different mechanisms leading to the activation of cellular protooncogenes have been discussed.

1. Mutation, either spontaneous or induced by chemical carcinogens or irradiation, leads to a structurally altered product of a cellular protooncogene that might provoke the changes underlying neoplastic transformation. This mechanism of activation has been demonstrated for the c-*ras* gene in several solid tumors and tumor-derived cell lines (6–8).

2. Chromosomal rearrangement leads to an enhanced expression of the protooncogene in question (9). Several protooncogenes have been mapped recently to chromosome regions, which are known to be preferential breakpoints involved with the cytogenetic alterations associated with some human and murine malignant diseases (for review see Ref. 10). Especially in human Burkitt's lymphoma and murine plasmocytoma specific rearrangement of the c-*myc* gene was shown (11, 12).

3. Amplification of a protooncogene leads to multiple copies of the gene being repeatedly arranged on the chromosomal DNA or extrachromosomally as double minutes. This mechanism of protooncogene activation also causes enhanced expression and has been observed with c-*myc*, L-*myc*, and N-*myc* (13–15).

The c-*src* gene, a protooncogene studied intensively with respect to its normal function (16–19), has not thus far been definitively associated with the causation and/or progression of human neoplasms. However, some intriguing observations have been made. The c-*src* gene in humans is located, as shown by *in situ* hybridization, on chromosome 20 (20). This chromosome is involved in several rearrangement processes associated with a variety of malignant blood diseases (21). Expression of c-*src* in such tumor cells was detected in a systematic study on leukemic children. In many patients hybridization of a *src*-specific probe to mRNA from tumor cells was reported, although in some samples the signal was only very weak (22). However, in the case of one T-cell acute lymphoblastic leukemia, one chronic myeloblastic leukemia, an osteosarcoma, and a Wilm's tumor the amount of c-*src*-specific mRNA was elevated 4–8-fold. In an acute myeloblastic leukemia the amount of c-*src* transcripts was enhanced as much as 8–16-fold. Similar studies have also revealed elevated levels of c-*src* mRNA in cells of acute lymphoblastic and acute myeloblastic leukemia patients as compared to normal cells (23) and in two patients with chronic myeloblastic leukemia (24).

A 4–20-fold enhanced enzymatic activity of the c-*src* gene product, a tyrosine-specific kinase, was found in some human sarcomas and mammary carcinomas (25).

In some human melanomas chromosome aberrations concerning chromosome 20 have been observed (26). Because c-*src* possibly could be involved in these rearrangements, its role in the process of melanoma formation either causative or secondary during tumor progression and metastases appears reasonable to investigate. The c-*src* gene product to be investigated might be changed qualitatively and/or quantitatively. Therefore, studying c-*src* expression on the mRNA level by Northern or dot blot analysis appears not to be sufficient, because qualitative changes leading to a more enzymatically active protein product or to a protein with altered substrate specificity would not be detectable. Thus, we decided to study c-*src* expression in human melanoma and other skin tumors by determination of its tyrosine-specific kinase activity, which reflects the ultimate gene action of the protooncogene. In addition we investigated the effect of the cytostatic drug DTIC[3] on the phosphorylating activity of the c-*src* protein product, the pp60$^{c\text{-}src}$, *in vitro*.

MATERIALS AND METHODS

Antisera. TBR sera were prepared by simultaneous injection of RSV Schmidt-Ruppin D and RSV Prague C strains into newborn rabbits by a modification (27) of the procedure described by Brugge and Erikson (28).

Tumor Material. A total of 12 skin metastases (in-transit or distant metastases of 8 patients (A–I) suffering from malignant melanomas (4 superficial spreading melanomas, 2 acrolentiginous melanomas, and 2 of nodular type) were investigated (Table 1). Patients A, D, E, and I were not subjected to cytostatic treatment during therapy prior to excision of the metastases. Patients B, F, G, and H bearing melanomas located on the limbs and exhibiting in-transit metastases were subjected to a twice repeated extracorporeal cytostatic perfusion. Studies on kinase activity in metastases after cytostatic drug therapy were performed on skin metastases which appeared on the treated limb only after the end of the therapy. The metastases were excised in local anesthesia and immediately frozen in liquid nitrogen or directly prepared on ice for the immunoprecipitation experiments.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: DTIC, (3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (dacarbazine); RSV, Rous sarcoma virus; TBR serum, antisera from RSV tumor-bearing rabbits; pp60, M_r 60,000 phosphoprotein; Ap4A, diadenosinetetraphosphate.

Table 1 *Characterization of the tumors investigated*

Patients	A	B	D	E	F	G	H	I
Age	45	72	36	72	70	69	60	62
Sex	M	F	M	M	M	F	F	F
Classification of primary tumor	NM[a]	SSM	SSM	SSM	NM	ALM	SSM	ALM
Level of invasion	V	IV	III	IV	V	IV	IV	V
Tumor thickness (mm)	5.0	2.6	2.5	3.6	19.0	2.2	3.3	ND
Location of primary melanoma	Right chest	Left calf	Left thigh	Epigastrium	Right hallux	Nail of right hallux	Left calf	Left hallux
Location of skin metastases	Right chest	Left thigh	Temple	Left chest	Right calf	Right thigh	Left thigh	Left calf
Grade of pigmentation	Heavy	Amelanotic	Amelanotic	Slight	Amelanotic	Amelanotic	Amelanotic	Amelanotic

[a] NM, nodular melanoma; SSM, superficial spreading melanoma; ALM, acrolentiginous melanoma; ND, not done.

Cell Lines. Cell lines used in this study were the established human melanoma cell lines SK-Me15 (29), RPMI 5966 (30), Colo 38 (31), 51/2, three different subclones (F9, E10, B12) of the primary melanoma cell line STR (established by K. H. Westphal), a human nevus cell line (established by K. Ax), and human foreskin fibroblasts. The cell lines were grown in Dulbecco-Vogt-modified Eagle's minimum essential medium supplemented with 10% fetal calf serum. Chicken embryo cells were prepared from 11-day-old embryos (Lohmann-Tierzucht GmbH, Cuxhaven, Federal Republic of Germany) and were maintained in Dulbecco-Vogt-modified Eagle's minimum essential medium containing 5% newborn calf serum. Secondary cells were infected with cloned virus of the Schmidt-Ruppin-A strain of Rous sarcoma virus.

Preparations of Cell Extracts and Immunoprecipitation. Tissue samples were lysed and clarified as described previously (16). The immunoprecipitation was carried out in antibody excess. Soluble protein (0.2 mg) was incubated with 5 μl TBR serum for at least 60 min at 4°C and precipitated with the protein A-bearing *Staphylococcus aureus* Cowan strain I. The bacterial-bound immunocomplex was washed and the protein kinase assay was carried out by a modification (16) of the method of Collett and Erikson (32). After 5 min the reaction was stopped. Further incubation did not increase the phosphorylation degree of the pp60^{src} immunocomplex. For the Ap4A and DTIC experiments, various concentrations of Ap4A and DTIC were added to the prewashed immunoprecipitates 5 min before the kinase reaction was started by addition of [γ-^{32}P]ATP.

Protein Determination. Determination of protein concentration in the supernatant of the clarified cell lysates was carried out on trichloroacetic acid-precipitated aliquots according to the method of Lowry *et al.* (33).

Phosphoamino Acid Analysis. ^{32}P-labeled IgG was cut out of the gel, eluted from the gel, and processed for phosphoamino acid analysis as described previously (16), following the method by Hunter and Sefton (34).

Isolation of DNA and Hybridization. DNA from human melanoma, from nontumorous tissue surrounding the tumor, and from peripheral blood lymphocytes of healthy persons was prepared according to the method of Blin and Stafford (35). Ten μg of either DNA were digested to completion with restriction enzymes *Eco*RI or *Hind*III, run on 0.8% agarose gels transferred to a hybridization membrane (Gene screen plus; NEN, Dreieich, Federal Republic of Germany) by the alkaline transfer procedure. The filters were hybridized to the nick-translated 600-base pair *src*-specific *Pst*I fragment F of clone SRA-2, encompassing the tyrosine kinase domain of the viral *src* gene (36). Hybridization was carried out at 43°C in a buffer containing 43% formamide and 5× standard saline citrate in the presence of 6×10^6 cpm ^{32}P-labeled probe (specific activity, 6×10^8 cpm/μg DNA). Subsequent washings were performed in 0.5× standard saline citrate at 63°C.

Histology. Part of the metastases were fixed in 4% formaldehyde and embedded in paraffin. Sections 8 μm thick were stained with hematoxylin-eosin. Photographs were taken on the Ortholux Orthoplan photomicroscope. Film material was Ilford PAN F.

RESULTS

Different types of human melanomas were analyzed for the presence and expression of c-*src*-related sequences (Table 1). A macroscopic view and stained sections of two melanomas are shown in Fig. 1. We determined the expression of the pp60$^{c\text{-}src}$ kinase activity by means of the immunocomplex kinase reaction in the presence of excess antiserum. All melanomas tested show a kinase activity which is reactive with pp60^{src} antibodies (Fig. 2). In any case the activity was elevated as compared to normal skin (Fig. 3). Quantitative determination of the kinase activity revealed varying kinase levels in the melanoma extracts of different patients and in the different metastases from the same patient (Fig. 3). A total of three different TBR sera were used throughout the experiments, all with the same results, whereas immunoprecipitates using a preimmune rabbit serum did not display significant kinase activity. While the melanoma of patient E showed a low kinase activity (compared to normal skin), the melanoma of patient I displayed kinase which is 5 times as active as that of patient E. Thus far no correlation between the pp60^{src} kinase activity and the pathological type of the melanoma is evident. Comparing different metastases of the same patient, we detected varying levels (factor of 2–3) of kinase activities in patients D and F but comparable activity in two metastases from patient G.

To investigate if cytostatic treatment during therapy has any effect on the pp60$^{c\text{-}src}$ kinase we compared the activity in different metastases from one patient before and after treatment. Thus far no difference in kinase activity with respect to cytostatic treatment was found (data not shown).

In addition to biopsy material we determined the kinase activity in several established melanoma cell lines (Table 2). These cell lines also showed varying kinase levels ranging from 1800 cpm/mg protein in SK-Me15 to 700 cpm/mg in 51/2 cells. As a control a nevus cell line was investigated. The nevus cellular nevus is a benign tumor arising from a localized, benign neoplasm of cells derived from epidermal melanocytes (37). The nevus cells are located either as foci in direct contact with the epidermis (junction nevi) or exclusively in the outer corium (dermal nevi). Since nevus cellular nevi are considered to be benign tumors of pigment cells, they are of the same histogenetic origin as malignant melanomas and are therefore more suitable as control tissue than is normal skin, the latter being of very heterogeneous composition. Unfortunately it was not possible to receive sufficient biopsy material from nevi. We therefore tested a nevus cell line for comparison, which interestingly displayed only low kinase activity, similar to the levels found in human foreskin fibroblasts (Table 2). Three different subclones of one primary cell line of a human melanoma were also tested and each showed pp60$^{c\text{-}src}$ kinase activity. The level of kinase activity, however, was different in each of the subclones. To confirm that it is indeed the heavy chain of the pp60^{src} antibody which is phosphorylated in the *in vitro* kinase assays, aliquots of each sample were run under nonreducing

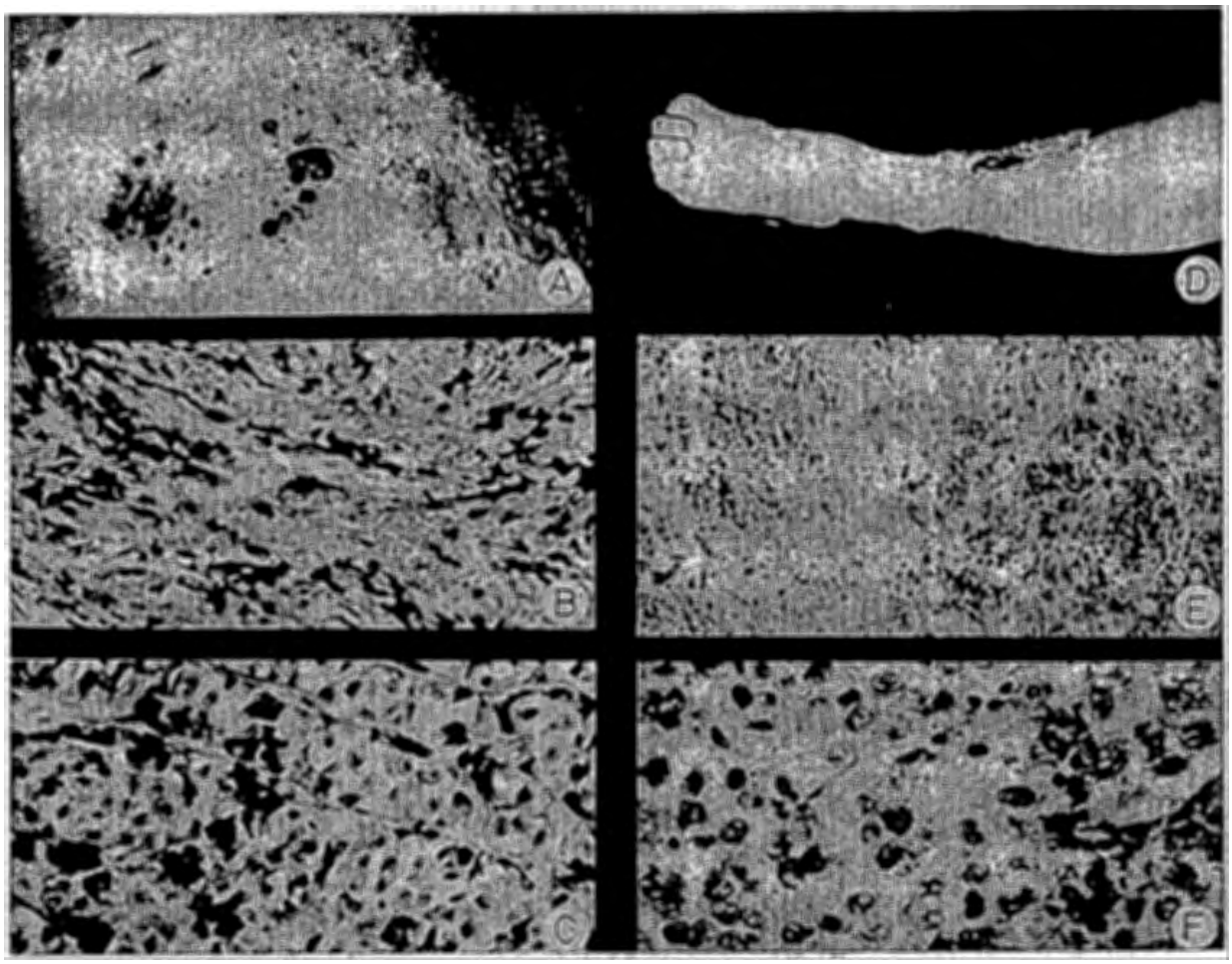

Fig. 1. Macroscopic and microscopic appearance of melanoma metastases. *A–C*, tumor A. *A*, multiple pigmented skin metastases of a nodular melanoma on the skin of the right chest of a 45-year-old male. *B*, *C*, histological picture of the metastases. Tumor cells in pseudoalveolar arrangement and multiple melanophages. *B*, × 110; *C*, × 260. *D–F*, tumor I. *D*, multiple amelanotic skin in-transit metastases of an acrolentiginous melanoma of the left hallux of a 62-year-old female. Recurrences 2-fold extracorporeal perfusion therapy. *E*, *F*, histological picture of the metastases. Large epithelioid tumor cells with polymorphous nuclei and many mitoses. *E*, × 110; *F*, × 260.

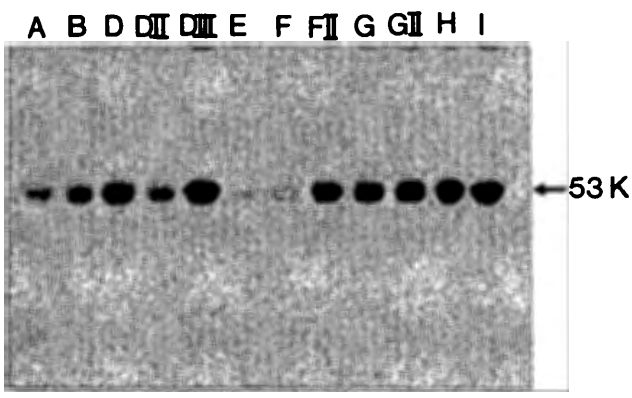

Fig. 2. Demonstration of the pp60$^{c\text{-}src}$ kinase activity in extracts of human melanomas. Equal amounts of protein were immunoprecipitated with a pp60$^{c\text{-}src}$-reactive TBR serum, the kinase assay was performed, and the proteins were separated on an 11% polyacrylamide gel. *53K*, heavy chain of the anti-pp60$^{c\text{-}src}$ immunocomplex. *DII* and *DIII* are different metastases of patient D, *FII* that of patient F, and *GII* that of patient G.

conditions and the radioactivity was then detected in the M_r 150,000 IgG band (data not shown). In order to establish that the IgG heavy chain phosphorylation was due to a tyrosine-specific kinase activity, we performed two-dimensional phosphoamino acid analyses; one example is shown in Fig. 4. The data indicated that there was only tyrosine phosphorylation. For further characterization we tested the kinase activity in the presence of Ap4A, a compound which exhibits an inhibitory effect on the viral pp60^{src} kinase activity but does not seem to affect the cellular enzyme in concentrations up to 100 μM (38). In all cases tested, the kinase activity was insensitive towards inhibition by Ap4A in concentrations ranging from 1–100 μM (data not shown). Thus far we have not detected any differences in the biochemical properties of the immunoprecipitated kinase activity from human melanoma compared to pp60$^{c\text{-}src}$ kinase activity from normal tissues from different species (16, 18).

Because the antitumor agent DTIC is widely used in melanoma therapy, we investigated whether this drug has any effect on the activity of the protooncogene product pp60$^{c\text{-}src}$. Addition of DTIC to the immunoprecipitates before starting the *in vitro* kinase assay led to a concentration-dependent inhibition of the tyrosine-specific kinase activity from human melanomas as well

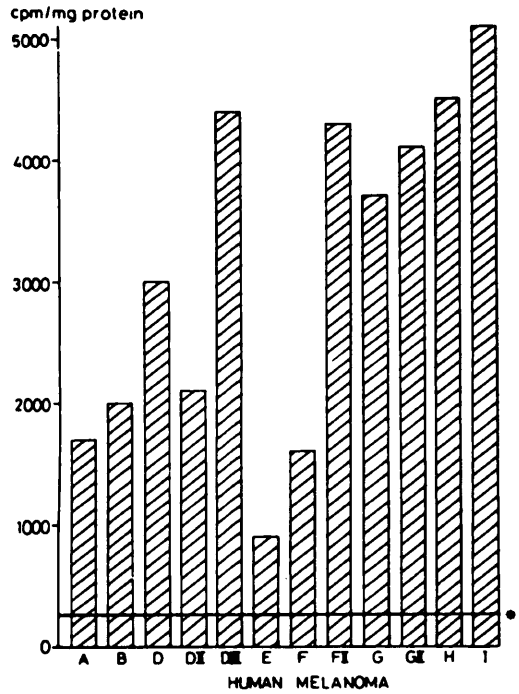

Fig. 3. Quantitative analysis of pp60$^{c\text{-}src}$ kinase activity in extracts of human melanoma. Experimental details as described in Fig. 2. After the gel was stained, the heavy chain IgG band was cut out of the gel, and the counts were eluted and counted in a liquid scintillation counter. *A–I*, melanomas from different patients. *DII*, *DIII*, different metastases from patient D, *FII* that from patient F, and *GII* that from patient G (•). As a control pp60$^{c\text{-}src}$ kinase activity was determined in extracts of normal human skin. Each sample was assayed 6 times. SD ±5%.

Table 2 *Quantitative determination of pp60$^{c\text{-}src}$ kinase activity in human melanoma cell lines*

Experimental details are as described in Figs. 2 and 3. SD ±5%.

Cell line	cpm/mg protein
SK-Me 15	1800
RPMI 5966	1300
Colo 38	1200
51/2	700
STR-F9	6000
STR-E10	4000
STR-B12	2000
Nevus cell line	300
Human foreskin fibroblasts	300

as the pp60^{src} kinase activity from RSV-transformed cells (Fig. 5). The concentrations of DTIC which led to a 50% inhibition of the kinase activity were 3.6 mg/ml for the viral kinase, 3.3 mg/ml for the cellular kinase from chick embryo cells, 1.6 mg/ml for the kinase from melanoma A, 2.8 mg/ml for the kinase from melanoma D, and 2.4 mg/ml for the kinase from melanoma I.

To determine whether the elevated kinase activity is a specific characteristic of melanoma or more characteristic of integumental cancers in general, we investigated a human skin tumor of a histogenesis different from that of melanoma. This tumor was excised from the right scapula of a 62-year-old patient (female) and was diagnosed as liposarcoma. After immunoprecipitation with TBR serum and subsequent performance of the kinase assay, we could show that the tumor cells display an increased kinase activity compared to the surrounding nontumorous adipose tissue of the same patient and to adipose tissue of a healthy person (Fig. 6). Elevation in the tumor was determined to be 8–10-fold.

To investigate whether the expression of c-*src* in human

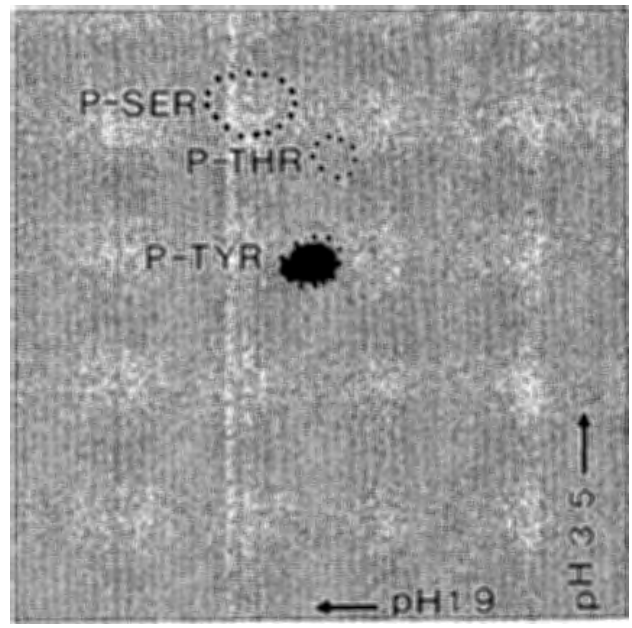

Fig. 4. Two-dimensional thin layer electrophoresis of ^{32}P-labeled heavy chain of TBR-IgG after precipitation of melanoma I extract. The ^{32}P-labeled heavy chain was eluted from the gel and hydrolyzed, and the phosphoamino acids were separated by electrophoresis at pH 1.9 in the first dimension and pH 3.9 in the second dimension. *P-SER*, phosphoserine; *P-THR*, phosphothreonine; *P-TYR*, phosphotyrosine.

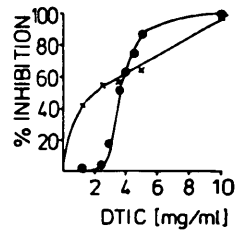

Fig. 5. Effect of DTIC on pp60$^{c\text{-}src}$ kinase activity immunoprecipitated from melanoma I extracts (×) and from pp60$^{v\text{-}src}$ kinase activity immunoprecipitated from Schmidt-Ruppin strain A RSV-transformed chick embryo cells (●). Experimental details are as described in Fig. 2 and "Materials and Methods."

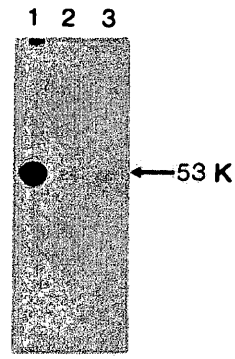

Fig. 6. Demonstration of the pp60$^{c\text{-}src}$ kinase activity in extracts of a human liposarcoma (*Lane 1*), nontumorous adipose tissue of the same patient (*Lane 2*), and adipose tissue of a healthy person (*Lane 3*). Experimental details as described in Fig. 2.

melanoma might be accompanied by gross structural changes in the c-*src* gene, we performed a Southern blot analysis of DNA isolated from human melanoma I which showed the highest levels of kinase activity. Hybridization to a v-*src*-specific probe revealed 19-kilobase restriction fragments in *Eco*RI di-

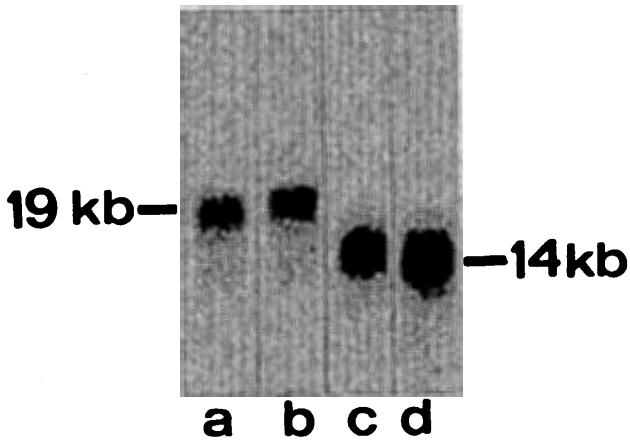

Fig. 7. c-*src* sequences in DNA isolated from melanoma I (*Lanes a* and *c*) and from peripheral blood lymphocytes (*Lanes b* and *d*). *Lanes a* and *b*, *Eco*RI-digested DNA; *Lanes c* and *d*, *Hind*III-digested DNA. For further details see "Materials and Methods." *kb*, kilobase.

gests and 14-kilobase fragments in *Hind*III digests (Fig. 7). DNA from peripheral blood lymphocytes of nontumorous persons showed the same intensity of bands and fragment sizes as the melanoma DNA (Fig. 7). The same applies for DNA from the nontumorous tissue surrounding the melanoma (data not shown).

DISCUSSION

$pp60^{c\text{-}src}$ tyrosine-specific kinase activity was detected in all melanomas examined, independent of their histological structure. The expression pattern of kinase activity in the melanomas tested is differential, and a high or low level of kinase activity seems more likely to be an individual feature of the single tumor case and not of a melanoma type in general. The finding that different metastases from the same patient show different levels of kinase activity tempted us to assume that c-*src* might be more involved in secondary changes during tumor progression than in the primary event of neoplastic transformation. This is also supported by the data on $pp60^{c\text{-}src}$ kinase activities in cell lines. Different lines are recognized by their individual kinase level. The differences found in three subclones of one primary melanoma cell line point to the interpretation that elevated kinase activity as observed in subclone F9 is acquired during the processes of tumor progression in some of the transformed cells and is not related to the event of neoplastic transformation in the cells giving rise to the primary melanoma. If the latter would be the case, all subclones should have similar levels of kinase activity.

The metastases from both the nodular melanomas showed a somewhat lower kinase activity than did the metastases from SSM and ALM.

The low kinase activity in patient E is probably due to the fact that in this case the tumor did not form a compact mass. Instead, diffuse nests of small nodules were found throughout cutaneous and s.c. tissues, so that the test material may have contained connective tissue in addition to tumor tissue.

The concentration-dependent inhibition of the $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ kinase activities by DTIC *in vitro* was also observed with tyrosine kinases coded by other RNA tumor viruses (data not shown). If DTIC has any effect on the $pp60^{c\text{-}src}$ kinase activity *in vivo* it might be a short term effect. We did not observe either in the DTIC-treated patients or in those who were subjected to cytostatic drug therapy other than DTIC a cytostatic effect on kinase activity in metastases excised 1-2 days following treatment.

We have shown that kinase activity is elevated considerably in some of the tumors and cell lines investigated. To evaluate the biological and pathological significance of that observation, several items must be taken into consideration.

1. Although it was clearly shown in some *in vitro* systems that elevated $pp60^{c\text{-}src}$ kinase activity alone is not sufficient to neoplastically transform cells (39, 40), a function during the process of tumor progression remains still possible. In that case a cooperation of c-*src* with one or more other activated protooncogenes in generating the neoplastic phenotype of the melanoma would be suggestive. For the human c-*ras* oncogene, it has been shown in several melanoma cell lines that this gene is activated through either point mutation or amplification (41, 42) and that the mutant c-*ras* is capable of neoplastic transformation of NIH3T3 cells.

2. The activation of c-*src* leading to increased kinase activity, which is observed in our tumor samples, does not seem to be melanoma specific, because in a skin tumor of different histogenesis also high levels of $pp60^{c\text{-}src}$ kinase activity were observed. This is in agreement with our earlier findings in the experimental animal system of *Xiphophorus*, where we detected elevated kinase activity in a variety of tumors of neuroectodermal and mesenchymal origin (43, 44) and with the data on enhanced c-*src* expression in some human blood cancers (23) and in some human sarcomas and mammary carcinomas (25).

3. At present we cannot decide whether the elevation of $pp60^{c\text{-}src}$ tyrosine kinase activity is due to enhanced c-*src* expression on the mRNA or protein level or to a structurally altered $pp60^{c\text{-}src}$ that displays higher levels of activity. Interestingly Bolen *et al.* (45) recently described in a human neuroblastoma cell line an increased $pp60^{c\text{-}src}$ tyrosine kinase activity which is associated with a unique amino-terminal tyrosine phosphorylation of the $pp60^{c\text{-}src}$ itself and which is not associated with high levels of c-*src* transcripts or $pp60^{c\text{-}src}$ protein. Whether this structurally altered $pp60^{c\text{-}src}$ would recognize cellular substrates other than those recognized by the $pp60^{c\text{-}src}$ from nontumorous cells is still unclear. In addition increasing $pp60^{c\text{-}src}$ kinase activity without an increase in the amount of c-*src* specific mRNA has been described recently during differentiation of HL-60 cells, a human leukemia cell line (46). In our first attempt to investigate the nature of the elevated kinase activity in human melanoma, we could not detect any gross structural changes in the c-*src* gene of the tumor showing the highest levels of kinase activity. Obviously, rearrangement and/or amplification of the c-*src* gene may not be responsible in general for the elevated kinase activity in human tumors.

The question if increased $pp60^{c\text{-}src}$ kinase activity in tumors is a cause or consequence of neoplastic transformation remains to be answered. A detailed study on c-*src* expression on the mRNA level including *in situ* hybridization, DNA transfection studies, and an extended biochemical analysis of $pp60^{c\text{-}src}$ from human melanomas should help to contribute to an understanding of the functional rate of $pp60^{c\text{-}src}$ in tumors.

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