

Identification of a Fish Protein Associated with a Kinase Activity and Related to the Rous Sarcoma Virus Transforming Protein¹

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ABSTRACT

Cells of all vertebrates thus far investigated contain a cellular *src* gene, which appears to be a highly conserved homolog of the Rous sarcoma virus oncogene *src*. Similar to the Rous sarcoma virus oncogene *src*, this endogenous cellular *src* gene codes for a *M*, 60,000 phosphoprotein (pp60^{c-src}) which is associated with a kinase activity. This kinase specifically phosphorylates tyrosine residues of proteins. We have systematically looked for pp60^{c-src} in specific strains of certain teleostean fish, including the genera *Xiphophorus* and *Girardinus*. These strains are known to contain chromosomal oncogenes causing neoplastic transformation in certain mutants and hybrids.

Since the pp60^{c-src} found in other species has been shown to be antigenically related to the viral *src* gene product, a phosphoprotein with a molecular weight of 60,000 (pp60^{v-src}), we have used tumor-bearing rabbit serum which recognizes Rous sarcoma virus pp60^{v-src} to detect such a related protein in fish. Tumor-bearing rabbit serum precipitated two proteins with molecular weights of 60,000 and 52,000. The *M*, 60,000 phosphoprotein was associated with a protein kinase activity that phosphorylates tyrosine residues of the immunoprecipitating immunoglobulin G heavy chain (*M*, 53,000). The antigenic relationship of this fish protein to viral pp60^{v-src} was further documented by an immune competition assay. We conclude, therefore, that the fish *M*, 60,000 phosphoprotein is a pp60^{c-src} encoded for by a fish cellular *src* gene that corresponds to the viral *src* oncogene. The *M*, 52,000 phosphoprotein is probably a proteolytic degradation product of pp60^{c-src}.

All animals of the various strains of the fish investigated in this study showed kinase activity that was reactive upon immunoprecipitation with tumor-bearing rabbit serum, indicating that the pp60^{c-src} protein is a normal constituent of the fish. Measurable quantitative differences were revealed between the various strains as well as in different tissues. Whereas brain and melanoma tissue showed a high kinase activity when compared to skin, liver, spleen, and testes, muscle tissue displayed no significant activity.

INTRODUCTION

In recent molecular biological studies on the origin and maintenance of the neoplastic state in vertebrates, emphasis is being placed on the identification of certain cellular genes of unknown function that correspond to the biochemically well-characterized oncogenes of RNA tumor viruses, such as *src* of

the RSV.³ All vertebrates thus far examined, including salmon, chicken, mouse, calf, and human, display nucleotide sequences in their genome, which are closely related to the sequences of the viral *src* oncogene and which are called *c-src* (23-26).

The viral pp60^{v-src} is intimately associated with a protein kinase activity which phosphorylates tyrosine residues of the heavy chain of IgG in immunoprecipitates (4, 6-9, 11).

A variety of cells from frogs, birds, and mammals have been shown to contain a protein, probably the gene product of *c-src*, pp60^{c-src}, that is closely related in structure and biochemical properties to pp60^{v-src} (3, 5, 16, 17, 22).

Although the homologies between the cellular and the viral oncogenes and gene products, respectively, suggest very similar biological functions, there is, to our knowledge, no convincing evidence to relate the *c-src* and its protein product to neoplasia induced by agents other than viruses. In order to demonstrate the possible involvement of the *c-src* or pp60^{c-src} in the nonviral induction of neoplasia, we have undertaken a new approach which is based on genetic and cytogenetic studies on the origin of neoplasia in certain teleostean fish. In *Xiphophorus*, a viviparous genus from Central America, and *Girardinus*, which is the corresponding genus from Cuba, we found that all individuals tested contain chromosomal genes that are essential for tumor formation (1, 21). Tumors occur spontaneously or following treatment with mutagens and/or tumor promoters in certain mutants and in interpopulational, interracial, and interspecific hybrids (2). It appeared worthwhile to investigate whether the expression of fish pp60^{c-src} is correlated to the expression of these fish oncogenes.

The present paper deals with the first part of our attempt to identify *c-src* genes in *Xiphophorus*, *Girardinus*, and some taxonomically related fish by means of quantitative studies on the protein kinase activity of pp60^{c-src}. In the second part, we will describe investigations correlating the activity of pp60^{c-src} with the phenotypic expression of the chromosomal oncogenes of the fish.⁴

MATERIALS AND METHODS

Experimental Animals

All fish used in the present study were bred in our laboratories under standard conditions (12). They were derived from geographically iso-

³ The abbreviations used are: RSV, Rous sarcoma virus; *c-src*, cellular *src* gene; pp60^{v-src}, *src* gene product, a phosphoprotein with a molecular weight of 60,000; pp60^{c-src}, cellular *src* gene product, a phosphoprotein with a molecular weight of 60,000; BC, backcross; SR-A, Schmidt-Ruppin strain A; SR-D, Schmidt-Ruppin strain D; TBR, tumor-bearing rabbit; pp52, phosphoprotein with a molecular weight of 52,000; pp60^{v-src}, viral *src* gene product, a phosphoprotein with a molecular weight of 60,000.

⁴ M. Scharf, A. Barnekow, H. Bauer, and F. Anders. Oncogenes in *Xiphophorus*: a relation in inheritability and expression of a tumor gene and the virus transforming gene, submitted for publication.

¹ This work was supported by the Sonderforschungsbereich 47 and 103 of the Deutsche Forschungsgemeinschaft.

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Received October 28, 1981; accepted February 19, 1982.

lated wild populations and were bred in closed stocks or inbred, respectively, for many generations. Neoplasia-developing hybrids were obtained by crossing procedures described earlier (1).

Inbred Fish

For denomination of taxa, see Refs. 18 and 19.

Poeciliidae. *Xiphophorus helleri*. Heckel 1848. Two populations were used: (a) Belize River (British Honduras), inbred since 1949, i.e., 80 generations; and (b) Rio Lancetilla (Mexico), inbred since 1951, i.e., 80 generations.

Xiphophorus maculatus. (Günther) 1866. Three populations were used: (a) Belize River (British Honduras), inbred since 1949, i.e., 80 generations; (b) Rio Jamapa (Mexico), inbred since 1939, i.e., 120 generations; and (c) Rio Usumacinta (Mexico), inbred since 1949, i.e., 80 generations.

Xiphophorus cortezi. Rosen 1960. Rio Axtla-Rio Panuco system (Mexico), probably inbred since 1939, i.e., 100 generations.

Xiphophorus variatus. (Meek) 1904. Two populations were used: (a) Rio Coy (Mexico), inbred since 1978, i.e., 10 generations; and (b) Rio Panuco (Mexico), no precise information about the year of capture available, inbred for more than 70 generations.

Girardinus falcatus. (Eigenmann) 1903. Central Cuba (particular locality of collection unknown). Loosely inbred for about 20 generations, subsequently highly inbred for 9 generations.

Girardinus metallicus. Poey 1854. Central Cuba (particular locality of capture unknown). Loosely inbred for about 20 generations, subsequently highly inbred for 11 generations.

Poecilia sphenops. Valenciennes 1846. Puerto Barrios (Guatemala), inbred for 15 generations.

Belonesox belizanus. Kner 1860. Chetumal, Yucatan Peninsula (Mexico) highly inbred for 8 generations.

Heterandria bimaculata. (Heckel) 1848. Ciudad Aleman (Mexico), inbred since 1978, i.e., 6 generations.

Goodeliidae. *Xenotoca eiseni*. Mexican Highlands (particular locality of capture unknown). Loosely inbred for about 25 generations.

Hybrids

BC hybrids of *X. maculatus* × *X. helleri* using *X. helleri* as the recurrent parent: (a) malignant melanoma-bearing BC hybrids and (b) benign melanoma-bearing BC hybrids.

Wherever the species is not taxonomically specified in the text, *X. helleri* was used.

Cells and Viruses

Chicken embryo cells were prepared from 9-day-old embryos (Lohmann, Cuxhaven, Federal Republic of Germany) and grown in Dulbecco's modified Eagle's medium (Flow Laboratories, Bonn, Federal Republic of Germany) supplemented with 5% calf serum. The cells were infected with SR-A of RSV. The virus-infected cells were maintained in the same medium supplemented with 1% dimethyl sulfoxide.

Antisera

Antisera from RSV tumor-bearing rabbits were prepared by simultaneous injection of SR-D RSV and Prague C strain RSV into newborn rabbits in a modification (27) of the procedure described by Brugge and Erikson (4).

Radioactive Labeling of Brain Cells

Brains of fish were chopped into small pieces (diameter, less than 0.5 mm), immediately washed once with prewarmed phosphate-buffered saline (0.17 M NaCl:3.3 mM KCl:10 mM Na₂HPO₄:1.8 mM KH₂PO₄, pH 7.2), and incubated at 28° for 3 hr with phosphate-free

Dulbecco's modified Eagle's medium containing 1 mCi of ³²P, per ml of medium.

Preparation of Cell Extracts and Immunoprecipitation

SR-A-transformed chick embryo cells (10⁷) were scraped off the plates with a rubber policeman in 1 ml phosphate buffer containing 10 mM sodium phosphate, 40 mM NaF, 10 mM EDTA, 1% Triton X-100, and 5% Trasylol (Bayer, Leverkusen, Federal Republic of Germany) as protease inhibitor. Fish samples were homogenized in the same buffer using a tightly fitting Dounce homogenizer (10 strokes at 4°).

The disrupted chicken and fish cells were centrifuged for 30 min at 6000 × g and then used immediately for immunoprecipitation. The clarified supernatants (1 ml) were incubated each with 5 to 10 μl of TBR serum for 60 min at 4°. A 10% suspension (30 to 50 μl) of formaline-fixed *Staphylococcus aureus* prewashed 3 times was added to absorb the immunocomplexes. After 30 min, the immunocomplexes were centrifuged for 2 min in an Eppendorf centrifuge, washed twice with 1 ml kinase washing buffer (10 mM sodium phosphate:40 mM NaF:10 mM EDTA:0.2% Triton X-100:1 M NaCl), and washed once with 1 ml H₂O. Unlabeled immunocomplexes were subjected to the kinase assay. Labeled samples were washed 4 times with kinase washing buffer and once with H₂O, and the pellet was then resuspended in 30 μl of sample buffer [2% sodium dodecyl sulfate:10% glycerol:70% H₂O:8% 1 M Tris (pH 6.8): 2% mercaptoethanol:0.02% 3',3'',5',5''-tetrabromophenol sulfonephthalein]. This mixture was boiled for 2 min and centrifuged for 2 min at 10,000 × g, and the supernatant was loaded onto a 11% acrylamide slab gel with a 2.5% acrylamide stacking gel. The labeled proteins were detected by autoradiography after staining, destaining, and drying of the gels. The molecular weight of the proteins on the gels was calibrated using ¹⁴C-labeled protein markers (Amersham Ltd., Amersham, United Kingdom) and ³²P-labeled heavy chain of IgG. For quantitation, the radioactive gel bands were cut out and solubilized, and their radioactivity was determined by liquid scintillation counting.

Protein Kinase Assay

The protein kinase assay was performed according to the method of Collett and Erikson (6), except that the incubation was at 4° for 5 min. Protein A-bound immunoprecipitates from unlabeled cell extracts were prepared as described above and suspended in 10 μl of kinase buffer (20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane:50 mM MgCl₂, pH 6.8) and 10 μl of approximately 0.1 μM [γ -³²P]ATP (>2000 Ci/mmol; Amersham Ltd.) in 50 μl of kinase buffer were added to each sample. The reaction was stopped by adding 1 ml quench buffer [100 mM sodium phosphate:10 mM EDTA:40 mM NaF:1 μM ATP (pH 7.0)] to the samples, and the labeled immunocomplexes were processed for gel electrophoresis as described above. Autoradiograms show equal amounts of protein for each sample. In a parallel experiment, the whole sample was loaded onto a gel, and the radioactivity in the heavy chain of IgG was determined as described above. Aliquots of the unlabeled samples were processed for protein determination.

Protein Determination

Determination of protein concentration in a supernatant of the centrifuged cell lysates was carried out on trichloroacetic acid-precipitated aliquots according to the method of Lowry et al. (14).

Phosphoamino Acid Analysis

³²P-labeled IgG of TBR serum-precipitated immunocomplex of fish brain extracts was cut out of the gel, eluted from the gel sample, and processed for phosphoamino acid analysis according to the method described by Hunter and Sefton (11) as modified by Ziemiński et al.⁵

⁵ A. Ziemiński, V. Bosch, F. X. Bosch, and R. R. Friis. Further analyses of several ts-td mutants of RSV showing separation of transformation parameters, submitted for publication.

Absorption Inhibition Assay

Heat-inactivated (30 min, 52°), SR-A-transformed chick embryo cell lysates were incubated with 2 μ l TBR serum diluted 1:160 for 12 hr on ice. Fish brain extracts were then added to the incubation mixture for 180 min. Subsequently, prewashed *S. aureus* was added, and the sample was processed for the protein kinase assay as described earlier. In a control experiment, the same amount of fish brain extract was directly incubated with the same amount of pp60^{src} antibodies for 180 min, and after addition of *Staphylococcus*, the kinase assay was performed.

RESULTS

Evidence of a TBR Serum-reactive M_r 60,000 Phosphoprotein in Fish. Fish brain tissue was radiolabeled with [³²P]P, *in vitro* and then extracted for immunoprecipitation with TBR serum. The precipitated proteins were analyzed by polyacrylamide gel electrophoresis. Two phosphoproteins, an M_r 60,000 protein, and an M_r 52,000 protein were detected (Fig. 1). Immunoprecipitation with normal rabbit serum did not show these proteins. Therefore, it is assumed that the precipitated M_r 60,000 phosphoprotein is the pp60^{src} correlate to the avian pp60^{src}. As the protease activity in brain is extremely high, the M_r 52,000 protein may be a degradation product of the M_r 60,000 protein. In the chick fibroblast RSV system, it has been shown that pp60^{src} is degraded into pp52 (10).

pp60^{src}-associated Kinase Activity in Brain Tissue. In a parallel experiment, the fish brain extracts were tested for a c-src kinase activity similar to the avian pp60^{src}-associated kinase. The strategy of these experiments was to measure phosphorylation of IgG in the TBR serum upon reaction with pp60^{src}-containing fish cell extracts. It was shown that IgG in this reaction was phosphorylated (Fig. 2), while immunoprecipitation with normal rabbit serum did not show phosphorylation of IgG. The phosphorylation occurred in tyrosine (data not shown), which is the specific amino acid residue phosphorylated by the RSV pp60^{src}-associated kinase (7, 11).

Comparison of pp60^{src} Kinase Activity in Different Tissues. Considerable differences in kinase activity were detected in the different tissues (Fig. 2). No significant activity was found in muscle. Skin, liver, and testes showed low activity as compared to that of spleen and benign melanoma, while in brain the kinase activity was always high. The highest kinase activity was found in extracts of malignant melanoma and of the brain of one and the same fish, whereas in fish with benign melanoma the melanoma and brain extracts both showed less kinase activity.

Absorption Inhibition Assay for pp60^{src} and pp60^{src}. To further confirm the antigenic relationship between the fish cell pp60^{src} protein and the pp60^{src}, an absorption inhibition assay was performed (Fig. 3). TBR serum was first reacted with heat-inactivated SR-A-transformed chicken cell extracts. These extracts did not show any kinase activity, but the antigenic sites remained intact as shown by Rübtsamen *et al.* (20). Subsequently, the pp60^{src}-specific serum was reacted with fish brain extracts. As shown in Fig. 3, the SR-A extracts were able to block TBR antibodies and to reduce the binding of the pp60^{src} antibodies to fish pp60^{src} by as much as 40%, indicating structural similarities between the fish pp60^{src} and the pp60^{src}.

Kinase Activity throughout the Lifetime of Fish. In order to

test whether the kinase activity changes during the lifetime of the fish, we determined the specific activity using the protein kinase assay in brain extracts and as a negative control in muscle extracts of *X. helleri* (Rio Lancetilla) of different ages. The kinase activity measured in neonates ($n = 30$) and 6-week-old ($n = 9$), 12-week-old ($n = 9$), 1-year-old ($n = 15$), and 2-year-old ($n = 5$) fish showed no significant differences.

Kinase Activity in Fish of Different Taxonomic Groups. No variation in the kinase activity between individuals of the same population was detected. In contrast, differences between individuals of the 2 fish families tested and between different genera (*e.g.*, *Heterandria* and *Girardinus*), different species (*e.g.*, *G. falcatus* and *G. metallicus*), and even different populations of the same species (*X. maculatus* from Belize River and from Rio Jamapa) could be demonstrated (Table 1). This variation is not correlated to the phylogenetic relationship or to the taxonomic differences of the fish tested in this study. The lowest amount of kinase activity was found in *P. sphenops* (80 cpm/mg protein) and the highest amount, in *X. helleri* from Rio Lancetilla (400 cpm/mg protein).

DISCUSSION

All vertebrates tested thus far contain endogenous genes related to viral genes which are responsible for the viral oncogenic capacity (13, 16, 17, 23). These cellular genes may therefore be called cellular oncogenes. Such an oncogene is the endogenous c-src which is a highly conserved homolog of src, the RSV-transforming gene.

In lower vertebrates, c-src was demonstrated so far only in salmon (25) and *Xenopus* (17), which have rather large genomes. It therefore appeared worthwhile to determine whether fish, such as *Xiphophorus* and related genera which have the basic genome of vertebrates (15), contain oncogenes such as c-src. Furthermore, *Xiphophorus* was especially attractive in a search for c-src as well as any other type of oncogene, because it is known from formal genetic studies that all individuals of all species and all populations of this genus contain a chromosomal oncogene that mediates neoplasia if it becomes derepressed (1, 2).

In all fish tested in this study, we identified a phosphoprotein that, as shown by its antigenic, biophysical, and enzymatic

Table 1
Expression of endogenous pp60^{src}-associated kinase in brain extracts of different fish species

Fish species	pp60 ^{src} -associated kinase
<i>X. helleri</i> (BR) ^a	+++
<i>X. helleri</i> (RL)	+++
<i>X. maculatus</i> (BR)	+++
<i>X. maculatus</i> (RJ)	++
<i>X. maculatus</i> (RU)	++
<i>X. cortezi</i>	+++
<i>X. variatus</i> (RC)	++
<i>X. variatus</i> (RP)	++
<i>G. falcatus</i>	+++
<i>G. metallicus</i>	+
<i>P. sphenops</i>	+
<i>B. belizanus</i>	++
<i>H. bimaculata</i>	++
<i>X. eiseni</i>	+++

^a BR, population Belize River; +++, 200 to 400 cpm/mg protein; RL, population Rio Lancetilla; RJ, population Rio Jamapa; ++, 100 to 200 cpm/mg protein; RU, population Rio Usamacinta; RC, population Rio Coy; RP, population Rio Panuco; + 50 to 100 cpm/mg protein.

properties, is related to the pp60^{src} of RSV. This fish phosphoprotein was detected by antiserum against pp60^{src}; it has an estimated molecular weight of 60,000 and is associated with a kinase activity which phosphorylates the tyrosine residue in the heavy chain of anti-pp60^{src} IgG. It is therefore assumed to be the product of a fish c-src gene. The M_r 52,000 phosphoprotein that has been detected together with the M_r 60,000 phosphoprotein after immunoprecipitation of ³²P-labeled fish brain extracts is assumed to be a proteolytic degradation product of pp60^{src} similar to that shown in the chicken system (10).

A certain level of kinase activity has been detected in skin, liver, spleen, and testes but not in muscle tissue. The highest level, however, has been found in melanoma and brain tissue. Since pigment cells, like brain cells, originate from the neuroectoderm, one can assume that the increased kinase activity, a common feature of both cell types, may be due to their ontogenetic relationship. On the other hand, the fact that brain cells do not proliferate, whereas melanoma cells show high rates of proliferation, indicates that elevated pp60^{c-src} kinase activity is not necessarily correlated with cell division. One possible explanation for the failure to detect significant amounts of pp60^{c-src} in muscle tissue is the inability to detect very low amounts of pp60^{c-src}. Another possibility could be that there is no expression of the c-src gene in fish muscle cells. It is worth mentioning in this context that the highest kinase activity found in fish is comparable to the activity found in normal chicken cells which, in turn, is about 30 to 50 times lower than in RSV-transformed cells (5). Furthermore, the proteins coded for by the various viral and cellular src gene homologs are not antigenically totally identical, so that a given antiserum must not detect all kinds of pp60^{v-src} or pp60^{c-src}, respectively.

The differences in kinase activity found in various organs of the fish, populations, species, genera, and families may be due to increased enzyme activity or increased gene expression. Thus far, no data allow us to differentiate between these 2 possibilities. Whether the elevated level of kinase activity in brain cells as compared to other nonneoplastic tissues points to a possible function of pp60^{c-src} essential for neural cells is not clear. In light of this, our finding of different degrees of kinase expression, depending on the fish genotype, appears of interest. Additional studies must be pursued in order to obtain further insights into the functional roles of pp60^{c-src} in neoplastic transformation and in normal cellular metabolism. A following paper will discuss possible genetic interrelationships between the c-src gene and a fish tumor gene.⁴

ACKNOWLEDGMENTS

We thank Dr. C. B. Boschek for critical reading of the manuscript. The excellent technical assistance of E. Ossendorf, U. Wend, and C. Reitz is gratefully acknowledged.

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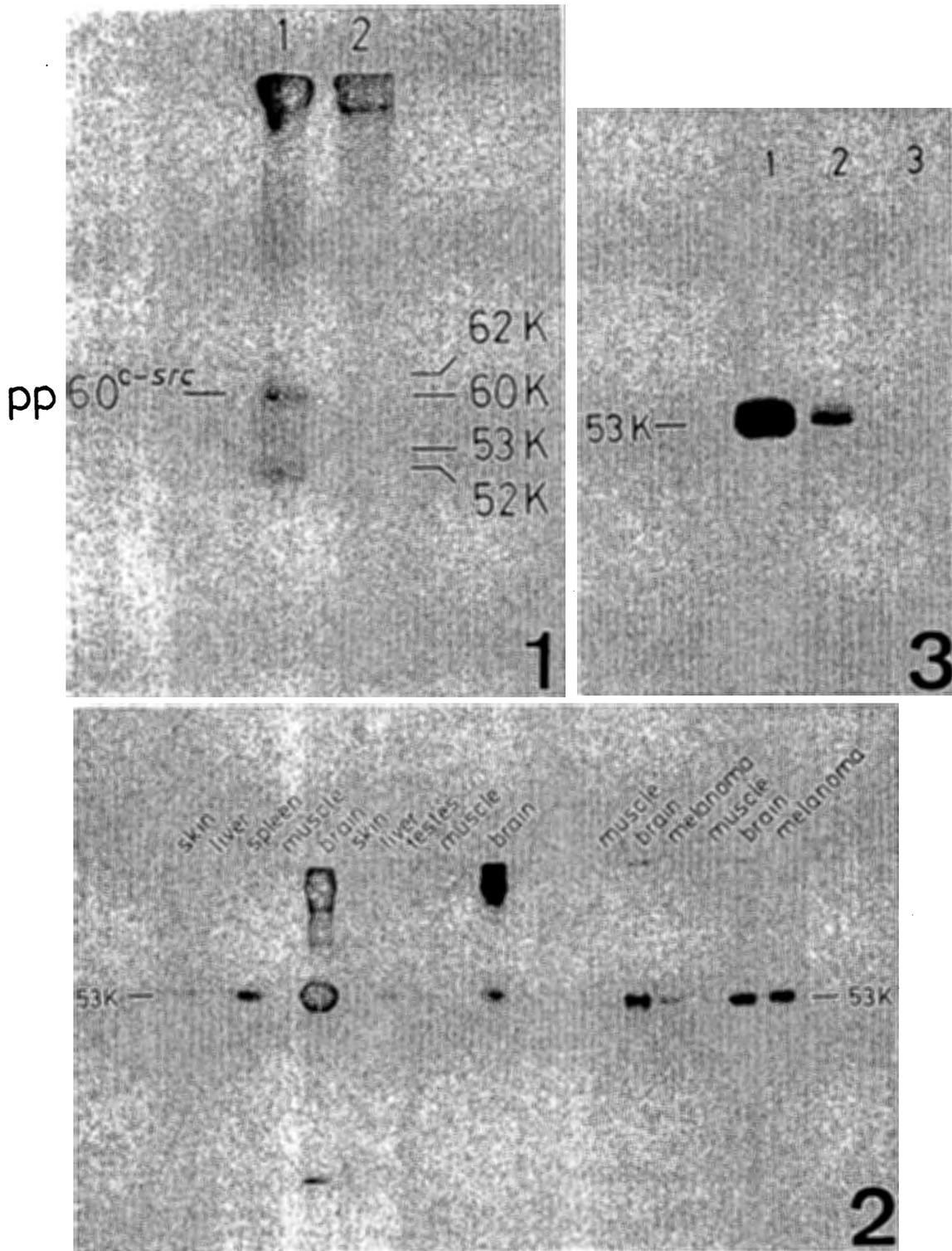


Fig. 1. Demonstration of ³²P-labeled pp60^{src} in fish brain extracts. Track 1, immunoprecipitation of fish brain extracts, labeled before with [³²P], *in vitro*, with TBR serum; Track 2, immunoprecipitation of the same extracts with normal rabbit serum. K, thousands.

Fig. 2. Demonstration of pp60^{src}-associated protein kinase activity in extracts of different fish organs. K, thousands.

Fig. 3. Absorption inhibition assay. Track 1, the pp60^{src} associated kinase activity in the immunoprecipitates of equal amounts of fish brain extracts and pp60^{src} antibodies; Track 2, the reduced pp60^{src}-associated kinase activity in immunoprecipitates of fish extracts with TBR serum after incubation with heat-inactivated SR-A cell extract; Track 3, immunoprecipitates of the heat-inactivated SR-A extract after incubation with TBR serum and processing the samples for the kinase assay. K, thousands.