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Autocrine Stimulation of the Xmrk Receptor Tyrosine Kinase in Xiphophorus Melanoma Cells and Identification of a Source for the Physiological Ligand*

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The melanoma-inducing gene of Xiphophorus fish encodes the Xmrk receptor tyrosine kinase. Using a highly specific antiserum produced against the recombinant receptor expressed with a baculovirus, it is shown that Xmrk is the most abundant phosphotyrosine protein in fish melanoma and thus highly activated in the tumors. Studies on a melanoma cell line revealed that these cells produce an activity that considerably stimulates receptor autophosphorylation. The stimulating activity induces receptor down-regulation and can be depleted from the melanoma cell supernatant by the immobilized recombinant receptor protein. The fish melanoma cells can thus be considered autocrine tumor cells providing a source for future purification and characterization of the Xmrk ligand.

In Xiphophorus, certain hybrid genotypes develop malignant melanoma at high incidence. Classical genetic analyses revealed that the dominantly acting tumor-locus (*Tu*-locus) is causally responsible for neoplastic transformation of pigment cells (Ahuja and Anders, 1976). Feral fish in their natural habitat are generally tumor-free due to the simultaneous presence of a tumor suppressor locus (*R*) which inactivates *Tu*. It is the crossing conditioned elimination of the *R*-locus which leads to the uncontrolled activity of the *Tu*-locus in the hybrid fishes resulting in the development of malignant melanomas.

The *Tu*-locus encodes the gene for a receptor tyrosine kinase (Xiphophorus melanoma receptor kinase, *Xmrk*), which was shown to be the melanoma-inducing oncogene of Xiphophorus (Wittbrodt *et al.*, 1989). Due to the loss of the suppressor, the *Xmrk* gene is deregulated in the hybrids. Its overexpression in pigment cells results in malignant melanomas (Adam *et al.*, 1993). Besides the oncogenic version of *Xmrk* from the *Tu*-locus, a second copy of *Xmrk* exists obviously representing the proto-oncogene (Schartl, 1990; Adam *et al.*, 1991). *Xmrk* codes for a protein with all of the structural features of subclass I receptor tyrosine kinases and shows highest similarity to the human EGF[1]-receptor (hEGF-R). It is a novel member of this gene family and thus is predicted to bind a so far unknown growth factor.

Receptor tyrosine kinases are a group of transmembrane cell surface receptors, which mediate their signaling function through the activation of an intracellular tyrosine kinase domain (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992). In the physiological situation, this activation results from the binding of the corresponding growth factor to the extracellular domain. The appropriate interactions of the ligand and the receptor warrant the normal function. Additionally several receptor tyrosine kinases appear as crucial effectors in the process of neoplastic transformation. Unscheduled activity of the tyrosine kinase domain is responsible for the proliferative capacity of the cancer cell (Ullrich and Schlessinger, 1990). In a few cases the activation of the tyrosine kinase activity was found to be due to activating mutations in the extracellular domain and/or the transmembrane domain, rendering receptor activity independent from the presence of the growth factor (Bargman and Weinberg, 1988; Roussel *et al.*, 1988; Woolford *et al.*, 1988). The most common cellular lesion in cancers where receptor tyrosine kinases are affected, however, is not mutational activation but receptor overexpression (Ullrich and Schlessinger, 1990). High receptor densities on cell surfaces could lead to activation via receptor oligomerization. On the other hand, many tumors and tumor-derived cell lines have been found to coexpress receptors and the corresponding growth factors (Sporn and Roberts, 1985; reviewed by Heldin *et al.* (1987)). This is interpreted as an autocrine mechanism of activation as proposed by Sporn and Todaro (1980). The functional significance of the various observations for the primary steps in tumor development remains to be elucidated. The Xiphophorus melanoma model offers a unique *in vivo* system to study the mechanisms involved in receptor tyrosine kinase-mediated tumorigenesis.

To understand how overexpression of the *Xmrk* oncogene leads to neoplastic transformation of pigment cells and thus to the development of malignant melanoma in Xiphophorus, analysis of the biochemical properties of the Xmrk receptor tyrosine kinase and the ligand/receptor interaction as well as the signal transduction pathway within the cell is required. We had shown previously that the Xmrk protein is indeed a functional receptor tyrosine kinase and that it carries an activating mutation in its extracellular or transmembrane domain which leads to a low level of constitutive tyrosine autophosphorylation. The intracellular kinase domain can be stimulated to high activities when fused to the extracellular ligand binding do-

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[1] The abbreviations used are: EGF, epidermal growth factor; hEGF-R, human EGF-receptor; TGFα, transforming growth factor; BHK, baby hamster kidney; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PLC, phospholipase C.

main of the human EGF-receptor and exposed to physiological concentrations of EGF or TGFα (Wittbrodt *et al.*, 1992). In the experiments reported here, we have expressed a recombinant Xmrk protein at high levels using the baculovirus system and produced a highly specific antiserum against this protein. Using these tools, we can demonstrate that the autophosphorylation of Xmrk is stimulated very much above the constitutive level by a factor present in melanoma cell culture supernatant. We conclude that the fish melanomá cells produce the physiological ligand of the Xmrk receptor in an autocrine manner and thus can be used as a source for the purification of this putative growth factor.

EXPERIMENTAL PROCEDURES

Generation of Recombinant Baculovirus—A deletion of all 5′-noncoding sequences was introduced in the Xmrk cDNA (pBII G4; Wittbrodt *et al.*, 1989) by *in vitro* mutagenesis (Bio-Rad Muta-Gene™ Phagemid *In Vitro* Mutagenesis Kit, Bio-Rad).

An oligonucleotide of 44 bases was synthesized with the 5′-part corresponding to bases 27–49 of the Xmrk cDNA (5′ CCGCGCAGAAACTCCATGAAGATCTAGAGCGGCCGCCACCGCGG 3′) and the 3′-part corresponding to bases 656–672 of the pBluescript KS II vector (Stratagene, La Jolla, CA). Thus, a deletion of 54 bases was introduced into pBII G4, creating a new unique *Bgl*II site at the point of deletion 5 bases upstream of the start codon (Fig. 1*A*). The entire coding sequence was isolated by restriction digest with *Bgl*II and *Dra*I and cloned into the *Bam*H I site of pAcYM1. Thus the plasmid pAcYM1mrk was derived (Fig. 1*B*).

Expression Constructs—For transient expression in human embryonic kidney fibroblasts (293 cells), the pRK5 Xmrk construct was used, whereas the CVN Xmrk construct was used for the production of the stably expressing baby hamster kidney cell line (BHK cells X12). Both plasmids have been described previously (Wittbrodt *et al.*, 1992).

Tissue Culture, Transfection and Infection, and Stimulation of Cells—The fish melanoma cells (PSM cells; Wakamatsu, 1981) were cultured at 28 °C in F-12 medium (Gibco BRL, Neu Isenburg, Germany), supplemented with 10% fetal calf serum (Seromed, Biochrom KG, Berlin) and antibiotics. To obtain conditioned medium, the subconfluent dishes were incubated with serum-free medium for 24 h. After this incubation the supernatant was immediately used for the stimulation experiments.

The human 293 embryonic kidney fibroblasts were cultured at 37 °C in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10% fetal calf serum and antibiotics. Transfection of these cells was done as described by Chen and Okayama (1987).

The baby hamster kidney cells (BHK X12) were cultivated in Dulbecco's modified Eagle's medium/F-12, 1:1, with 10% fetal calf serum and antibiotics at 37 °C.

The *Spodoptera frugiperda* Sf9 cells were cultured at 28 °C in TC100 medium (Gibco BRL). Maintenance and infection of these cells were done according to Summers and Smith (1988). The standard infection with the baculovirus was done at a multiplicity of infection of 10 in 175-cm^2 flasks.

For stimulation, transfected 293 cells or the BHK cells were grown as monolayers on six-well plates. The medium was removed and substituted with 0.5 ml of conditioned medium. The incubation was routinely done for 10 min at 37 °C and 5% CO_2. The cells were lysed immediately afterwards, as described below.

Tryptic Digestion—MNPVmrk-infected Sf9 cells were collected 68 h post-infection by low speed centrifugation at 1500 × *g* for 5 min, washed once with phosphate-buffered saline (PBS), and then resuspended in PBS. Trypsin and EDTA were added to final concentrations of 0.05 and 0.02%, respectively. After incubating for 10 min at room temperature, the reaction was stopped by adding fetal calf serum to a final concentration of 20%. The cells were pelleted as above, washed with PBS, and finally resuspended in lysis buffer and lysed as described below.

Metabolic Labeling of Cells—To metabolically label proteins, cells were transferred to labeling medium (methionine-free Dulbecco's modified Eagle's medium, Gibco BRL) supplemented with 1% dialyzed fetal calf serum and antibiotics. 50 μCi/ml of [^{35}S]methionine (Amersham, Braunschweig, Germany) was added. Cells were cultured in this medium overnight and subsequently lysed as described below.

Cell Lysis—MNPVmrk infected Sf9 cells were collected 68–70 h post-infection by low speed centrifugation (1500 × *g*, 5 min). The cell pellet was washed twice with PBS and then resuspended in lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin), at a density of 1 × 10^7 cells/ml. As the Xmrk-expressing Sf9 cells were resistant to osmotic or moderate mechanical lysis procedures, the cell suspension was passed through a French press (SLM Aminco minicell, at a maximum pressure of 20,000 p.s.i.). PSM and 293 cells were lysed with the same buffer on the culture dishes by incubating on ice for 5 min. The supernatant was then collected and incubated another 5 min before removing the cell debris by centrifugation at 14,000 × *g* for 5 min. For analyzing tyrosine phosphorylation, 100 mM NaF and 0.2 mM sodium orthovanadate were added to the lysis buffer.

Immunization of Rabbits—Rabbits were immunized with the recombinant Xmrk protein produced in MNPVmrk-infected Sf9 cells. The antigen was cut out of a Coomassie-stained preparative SDS gel, the gel slices were fragmented by passing through a metal mesh and an emulsion with an equal amount of Freund's adjuvant was formed by repeatedly passing the 1:1 mixture through an injection needle coupled to two syringes. For the primary injection, complete Freund's adjuvant was used, incomplete adjuvant was used for all subsequent injections. One-hundred μl of the emulsion were injected subcutaneously under each leg of the rabbit and an intramuscular injection of 300 μl was placed into the muscle of each rear leg. Secondary boosts were done at intervals of 6 weeks, and the bleeding was done 10 days after the boosts. For all experiments described in this manuscript the serum αmrk (K.2) was used without further purification procedures.

Immunoprecipitation—For immunoprecipitation, lysates were diluted 1:3 in HNTG buffer (20 mM Hepes pH 7.2, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) containing phosphatase inhibitors (100 mM NaF and 0.2 mM sodium orthovanadate). Twenty μl of protein A-Sepharose (Pharmacia, Freiburg, Germany) in a 1:1 slurry in HNTG and the appropriate amount of antiserum (either 5 μl of the polyclonal αmrk serum or 1 μl of the monoclonal αPY antibody) were added and incubated at 4 °C for at least 3 h. The Sepharose was pelleted by brief centrifugation (5 s, 14,000 × *g*) and washed three times with HNTG. For SDS-PAGE analysis 50 μl of sample loading buffer were added.

The anti-phosphotyrosine antibody (αPY) is a monoclonal antibody (clone 5E.2), which detects phosphorylated tyrosine residues.

Analysis of Glycosylation—Lysates of MNPVmrk-infected Sf9 cells were immunoprecipitated with the αmrk serum, the immunoprecipitates were washed twice with HNTG buffer, and the Xmrk protein was eluted from the Sepharose by boiling in 30 μl of elution buffer (50 mM Tris/HCl pH 6.8, 0.5% (w/v) SDS, 0.1 M β-mercaptoethanol) for 5 min. To 10 μl of the eluate, 5 μl of endoglycosidase H buffer (200 mM sodium citrate, pH 5.5), 5 μl of endoglycosidase H (1 milliunits/μl, Boehringer, Mannheim, Germany), and phenylmethylsulfonyl fluoride to a final concentration of 1 μg/μl were added. After an incubation at 37 °C for 16 h, the proteins were analyzed by SDS-PAGE.

Western Blotting—Using 0.2 M phosphate buffer, the proteins were electroblotted to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) by standard procedures. The membranes were blocked for 1 h at room temperature with NET-G (150 mM NaCl; 5 mM EDTA; 50 mM Tris/HCl, pH 7.4, 0.05% Triton X-100; 0.25% gelatin) with several changes of the buffer. After incubating filters with the primary antibody for 6 h at 4 °C and extensive washing for another hour (room temperature), the filters were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies for 1 h at 4 °C and then washed again as above. The detection of bound antibodies was done with the nonradioactive ECL system (Amersham).

Preparation of the Ligand Affinity Matrix—The glutathione *S*-transferase/SH2 fusion protein (GST-SH2) was produced in *Escherichia coli* XL1blue transformed with the expression construct I (Rotin *et al.*, 1992), containing nucleotides 1697–2633 of the human PLCγ, which comprises the two SH2 and the SH3 domains. Isolation of the fusion protein was done as described by Rotin *et al.* (1992). It was purified by adsorption to glutathione-Sepharose (Pharmacia), and stored as a suspension in PBS. The recombinant Xmrk receptor was bound to this matrix by incubation of the Sepharose with the cleared lysate of infected Sf9 cells at 4 °C for 2 h. The Sepharose was then pelleted by low speed centrifugation and washed three times with PBS. For the depletion experiment, the Sepharose was incubated with the conditioned medium for 30 min at 4 °C. The supernatant was separated from the Sepharose by centrifugation and used in the stimulation experiment.

RESULTS

High Expression of Recombinant Xmrk—To obtain a recombinant baculovirus that expresses the *Xmrk* gene, a full-length cDNA of the oncogene transcript (Wittbrodt *et al.*, 1989) was

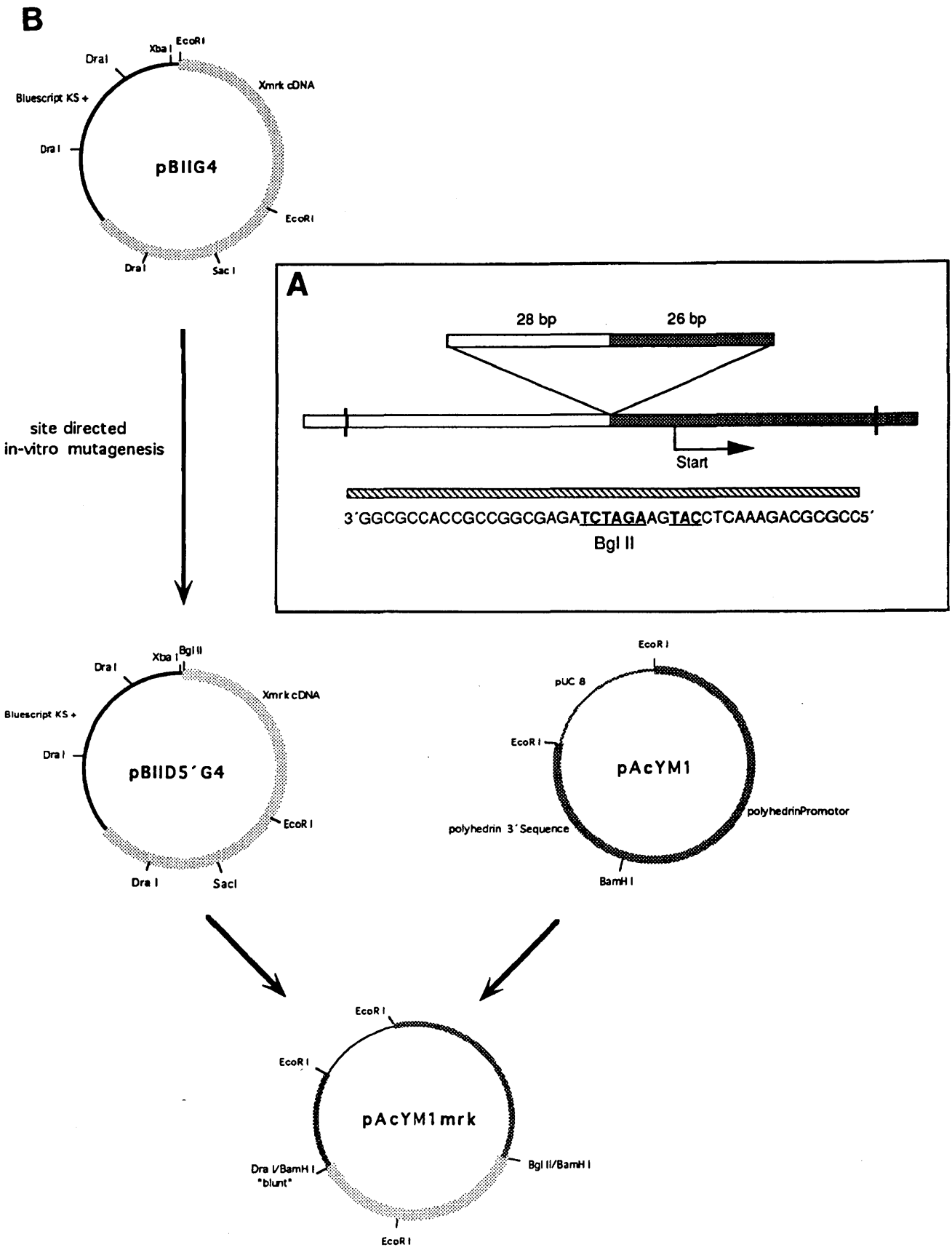

FIG. 1. **Construction of the baculovirus expression vector.** *A*, site-directed *in vitro* mutagenesis: schematic representation of the mutagenized 5'-untranslated region of the Xmrk cDNA. ■, Xmrk cDNA; □, Bluescript KS+; ▨, oligonuclotide. *B*, cloning strategy for the baculovirus expression vector pAcYM1mrk.

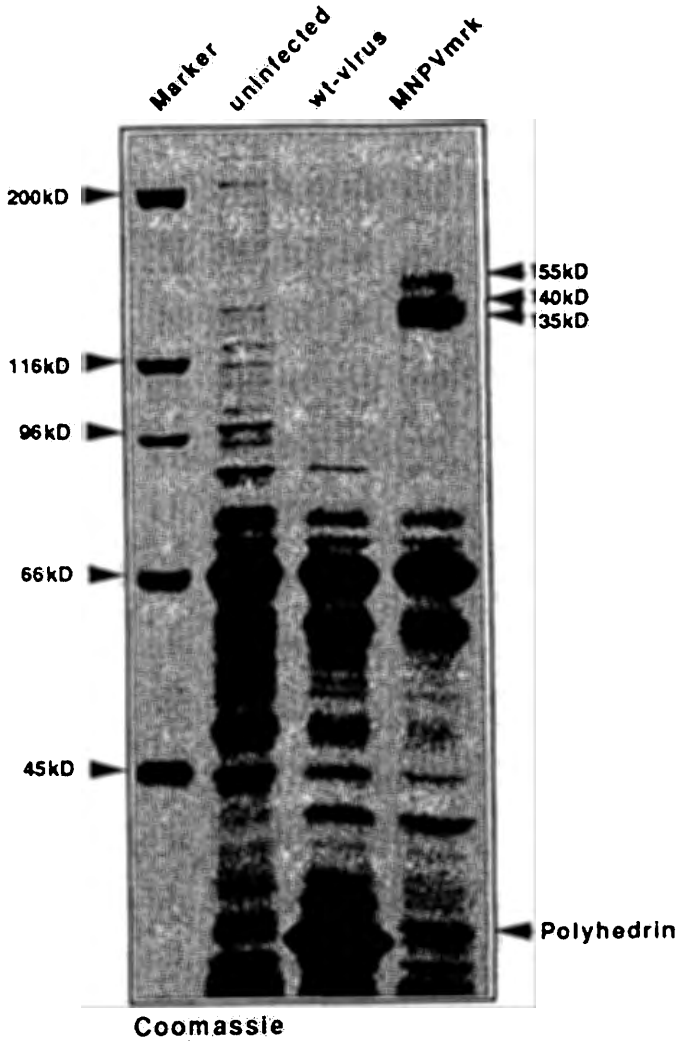

FIG. 2. **Expression of Xmrk in MNPVmrk-infected Sf9 cells.** 1×10^5 MNPVmrk-infected cells were lysed in lysis buffer, cell debris was removed by centrifugation, and the supernatant was separated on an 7.5% SDS-polyacrylamide gel. As controls uninfected or wild type virus-infected cells were used. The proteins were stained with Coomassie Brilliant Blue.

cloned into the plasmid vector pAcYM1 (Matsuura *et al.*, 1987). As the distance between the viral polyhedrin promotor and the translational start is known to be a critical parameter to gain optimal expression (Rankin *et al.*, 1988; Luckow and Summers, 1989), all 5'-noncoding sequences of the X*mrk* cDNA were deleted. This was achieved by introducing a suitable cloning site (*Bgl*II) two nucleotides upstream of the start codon by oligonucleotide-directed *in vitro* mutagenesis (Fig. 1*A*). The resulting plasmid pAcYM1mrk (Fig. 1*B*) was cotransfected together with wild type AcMNPV DNA into Sf9 cells. Recombinant viruses (MNPVmrk), where homologous recombination between sequences of the polyhedrin gene in the plasmid and the wild type gene had occurred, were isolated and purified by two rounds of plaque assay by taking advantage of a polymerase chain reaction screening technique (Malitschek and Schartl, 1991).

Expression of Xmrk in insect cells infected with MNPVmrk was analyzed by SDS-PAGE. Three additional proteins could be detected in whole cell lysates of infected cells, which were absent in uninfected or wild type infected cells (Fig. 2). According to estimations from Coomassie-stained gels, they made up about 40% of the total cellular protein. The size of these proteins was determined to be 155, 140, and 135 kDa. When separating the proteins on minigels (8 cm × 7 cm, Biometra), the

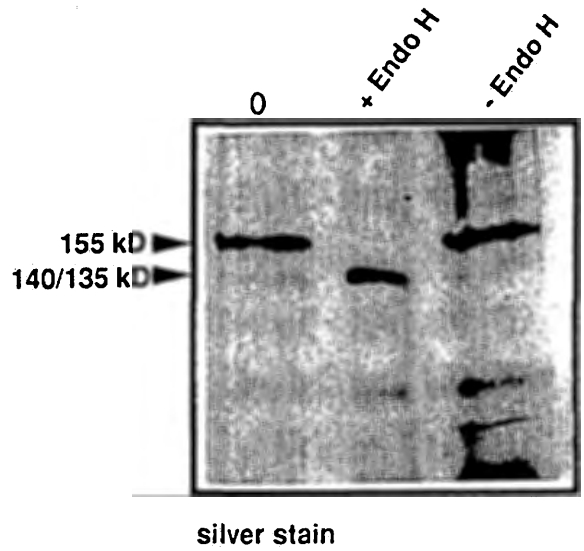

FIG. 3. **Analysis of the glycosylation of recombinant Xmrk.** The receptor was immunoprecipitated from the lysates of MNPVmrk-infected Sf9 cells with the αmrk antiserum and eluted from protein A-Sepharose. The eluted protein was digested with endoglycosidase H and then analyzed by SDS-PAGE. As controls an aliquot of the eluate (*0*) and a sample where no enzyme had been added (*–Endo H*) were used. On this gel, the 135- and the 140-kDa protein could not be separated. The proteins were detected by silver staining.

135- and 140-kDa protein comigrated as a single band. The predicted size of a nonposttranslationally modified Xmrk protein is in accordance with the smallest of the recombinant proteins. After treatment of the proteins with endoglycosidase H, only the 135-kDa protein could be detected (Fig. 3). Thus, the 155-kDa protein obviously is a glycosylated form of the recombinant Xmrk protein in which some or all of the predicted 12 *N*-glycosylation sites in the extracellular domain are modified. To analyze the subcellular localization of the recombinant protein, MNPVmrk-infected cells were treated with trypsin prior to lysis. Two proteins of approximately 100 and 60 kDa were the major products of this digestion (Fig. 4). The sizes of these digestion products are in perfect agreement with those expected for the correct localization of Xmrk as a transmembrane receptor. Cleavage at the trypsin site adjacent to the transmembrane domain would leave a predicted Xmrk fragment of 59 kDa consisting of the transmembrane domain and the intracellular part of the receptor. The 100-kDa fragment can be regarded as the product of a partial digest at another extracellular trypsin site.

Abundant Expression of Xmrk in Melanoma—An Xmrk specific antiserum (αmrk) was produced using the recombinant Xmrk protein from AMNVmrk-infected Sf9 cells. With this polyclonal serum, it was shown that the Xmrk protein is very abundant in lysates of a melanoma-derived fish cell line (PSM cells), which overexpresses the X*mrk* gene. The antiserum detects the 160-kDa mature receptor, as well as a 155-kDa underglycosylated intracellular precursor (Fig. 5 and data not shown). This antiserum was tested for cross-reactivity with the human EGF-receptor (hEGF-R). PSM cells and the human carcinoma cell line (A431), which overexpress the EGF receptor, were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with the αmrk serum. In PSM cell extracts, the αmrk serum precipitates both forms of the receptor (Fig. 6). No cross-reactivity of the serum with the hEGF-R could be detected in the lysates of the A431 cells (Fig. 6). On immunoblots of total cellular extracts of various fish tissues, the αmrk serum detected Xmrk as a very abundant protein in melanoma biopsies. The receptor could not be detected by these immunological methods in any of the normal tissues tested (Fig. 7*A*).

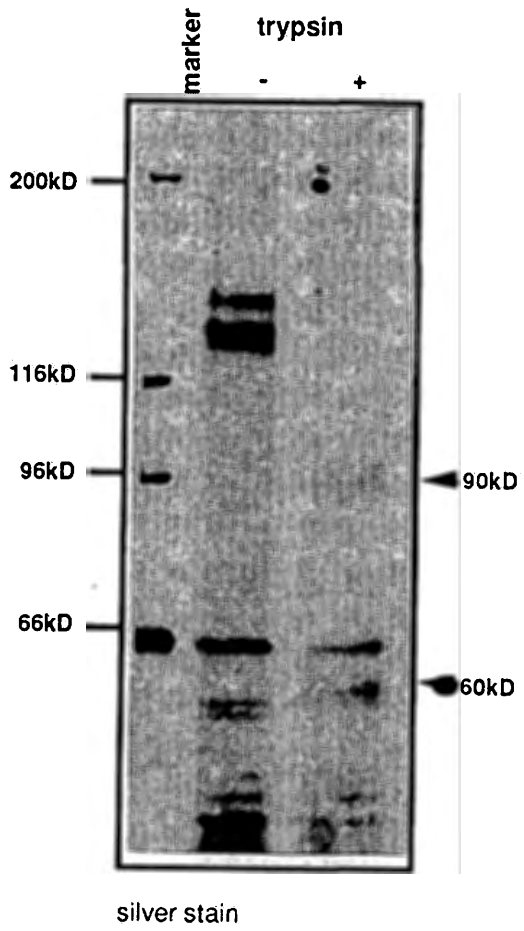

FIG. 4. **Transmembrane localization of Xmrk expressed in Sf9 cells.** 1×10^5 MNPVmrk-infected cells were treated with trypsin prior to lysis. The cleared lysates of these cells were analyzed by SDS-PAGE. Proteins were detected by silver staining. As a control, lysate of untreated cells was used.

Receptor Phosphorylation—The physiological activity of receptor tyrosine kinases is generally reflected by the state of phosphorylation of carboxyl-terminal tyrosine residues. To investigate the state of activation of the Xmrk receptor, the phosphorylation on tyrosine was examined using a monoclonal antibody directed against phosphotyrosine residues (αPY). We found that the Xmrk protein is the most abundant tyrosine phosphorylated protein in melanoma biopsies (Fig. 7*B*). In the melanoma cell line (PSM cells), the mature 160-kDa receptor proved to be tyrosine-phosphorylated in contrast to the underglycosylated 155-kDa form (Figs. 5 and 6). In the Sf9 cells we found tyrosine phosphorylation on all isoforms of the receptor, but predominantly on the high molecular weight form (Fig. 5).

Stimulatory Activity in Melanoma Cell Culture Supernatants—To address the question whether the receptor activation in PSM cells may be due to some ligand mediated autocrine stimulation, we incubated Xmrk-expressing human 293 cells with PSM conditioned medium prior to lysis (Fig. 8). A significant enhancement of the tyrosine phosphorylation of the receptor compared with untreated cells or cells incubated with non-conditioned growth medium was observed. The same stimulation was detected in BHK cells stably expressing Xmrk. A time course experiment revealed that this activation is maximal after 20 min of incubation and decreases at incubation times of more than 30 min (Fig. 9).

To show that the stimulating activity in the supernatant can bind specifically to the Xmrk receptor, we depleted this activity from the conditioned medium using the recombinant receptor

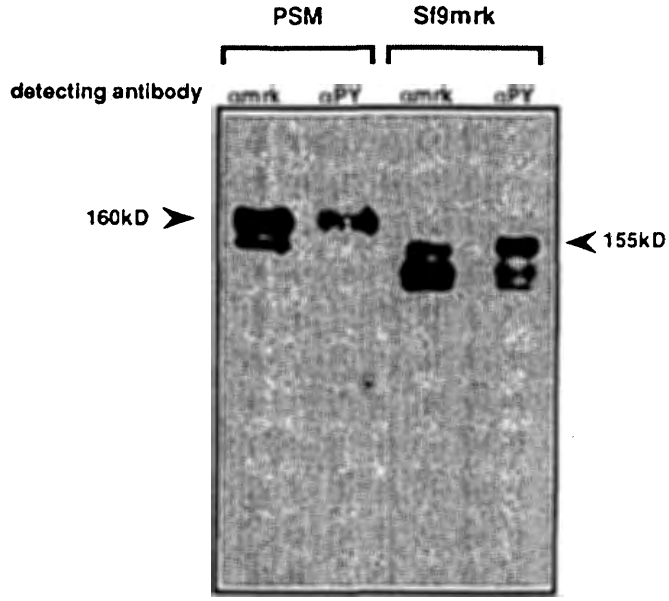

FIG. 5. **Tyrosine phosphorylation of different receptor isoforms.** PSM cells and MNPVmrk-infected Sf9 cells were lysed in Laemmli buffer, separated on 7.5% polyacrylamide gels, and blotted. The blots were immunoprobed with αmrk serum or the αPY antibody.

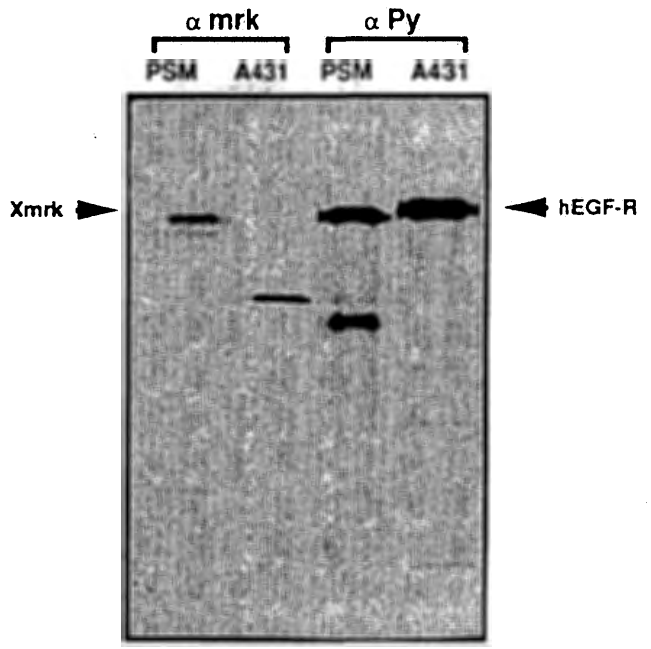

FIG. 6. **Immunoprecipitation of Xmrk or hEGFR with αmrk or αPY antibody.** PSM cells or A431 cells were labeled *in vivo* with [^{35}S]methionine and lysed. The lysates were immunoprecipitated with αmrk or αPY. The precipitates were analyzed by SDS-PAGE. In the human cell line, the αmrk serum precipitates a protein of 130 kDa, which could not be identified so far.

as an affinity matrix. To immobilize the receptor, we used a glutathione *S*-transferase (GST) fusion protein with the SH2 domain of the human PLCγ (Rotin *et al.*, 1992). It has been shown that the SH2 domain of PLCγ binds tightly to Xmrk by interacting with the COOH-terminal autophosphorylation sites.[2] The fusion protein was attached to the glutathione-Sepharose, and the receptor was then absorbed to this matrix. By incubating the cell culture supernatant with this matrix prior to the stimulation experiment, the stimulatory activity was depleted very efficiently. Depletion with GST-SH2 matrix

[2] C. Wellbrock, personal communication.

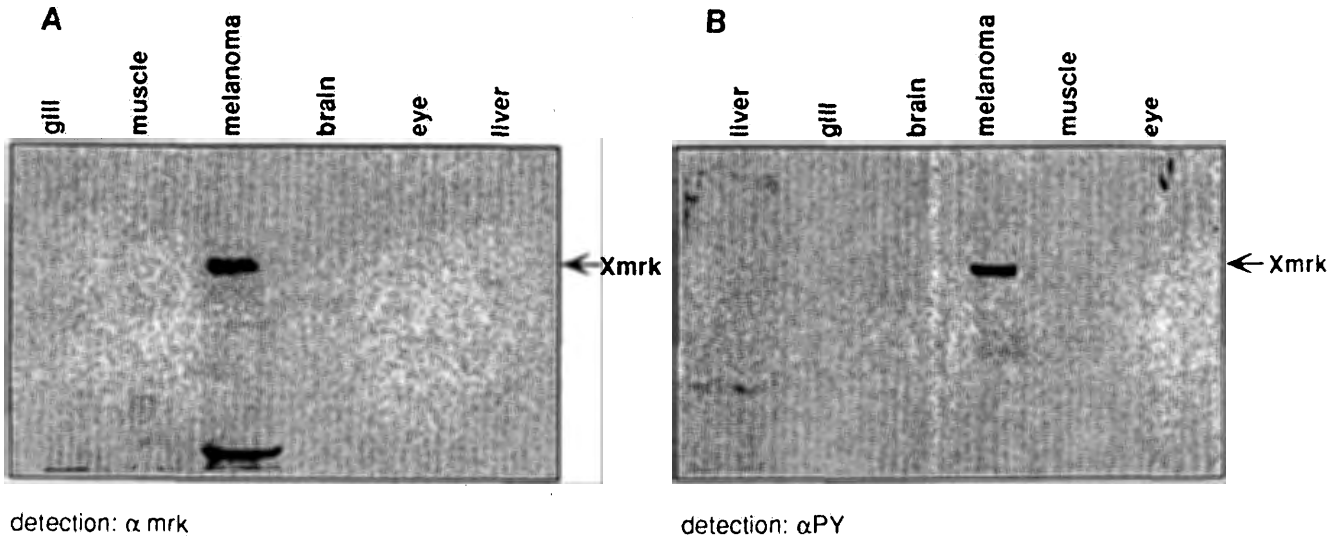

FIG. 7. **Overexpression and activation of the Xmrk receptor in fish melanoma.** Cell lysates of tissue biopsies were prepared and separated on SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membrane and detected with either the αmrk serum (*A*) or the αPY antibody (*B*).

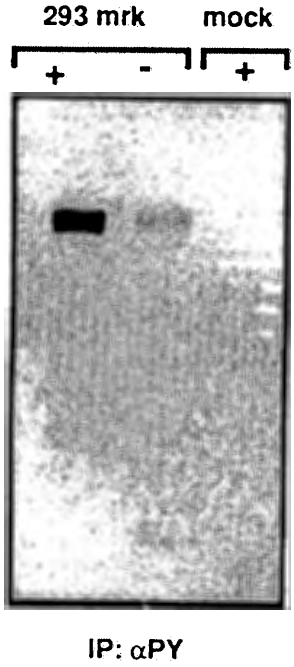

FIG. 8. **Stimulation of receptor autophosphorylation with conditioned medium of the melanoma cell line (PSM).** Xmrk and mock-transfected 293 cells were metabolically labeled with [^{35}S]methionine and either treated with PSM-conditioned medium (+) or with pure growth medium (–) prior to lysis. The lysates were immunoprecipitated with αPY and the precipitates were analyzed by SDS-PAGE.

alone (*i.e.* without Xmrk receptor) showed no negative effect on the stimulation (Fig. 10).

DISCUSSION

In order to characterize the biochemical functions and signaling properties of the Xmrk receptor tyrosine kinase as well as to approach the identification of its ligand, we have overexpressed the protein using the baculovirus expression system. We chose the baculovirus system, because it has proven to be very efficient for the expression of eukaryotic proteins and particularly for transmembrane receptors (Greenfield *et al.*, 1988). The Xmrk protein expressed in MNPVmrk-infected Sf9 cells makes up about 40% of the total cellular protein. This is very close to the optimum level of protein expression that can be expected with baculovirus vectors. The polyhedrin product of the gene whose promotor we used for expression accumulates to a level of about 50% of the total cellular protein level in wild type virus-infected cells (Luckow and Summers, 1988). In our attempts to analyze the Xmrk protein expressed in the Sf9 cells, we could show that the signal peptide of the fish receptor is obviously correctly recognized and that most of the produced protein is integrated into the plasma membrane of the insect cells. This led to a very high protein content in the membrane, which rendered the infected cells very resistant to standard lysis procedures.

It has been shown that *N*-glycosylated proteins produced in insect cells are of the high mannose type and that conversion to complex *N*-linked oligosaccharides generally does not occur (reviewed by O'Reilly *et al.* (1992)). This would explain the observed size difference of 5 kDa between the native Xmrk from fish melanoma cells and the recombinant Xmrk protein expressed in Sf9 cells. By digestion of the recombinant Xmrk protein with endoglycosidase H, it was demonstrated that the 155-kDa and very likely the 140-kDa species as well are partially glycosylated forms of the high mannose type and that the 135-kDa species is the unglycosylated precursor. In the PSM cells, we also found a high mannose type precursor of the receptor with a size of 155 kDa, which is not integrated into the membrane and subsequently is not phosphorylated. The shift in electrophoretic mobility of the mature receptor to an apparent molecular weight of 160,000 is obviously due to the processing of the high mannose chains to complex oligosaccharides, similar to what has been observed for the EGF receptor (Mangelsdorf Soderquist and Carpenter, 1984). The tyrosine phosphorylation of the recombinant receptor in the insect cells is very likely caused by the extreme overexpression and the very high receptor density in the membrane of the expressing Sf9 cells, which would result in a ligand independent transphosphorylation of the receptors.

With the αmrk serum, the Xmrk protein was detected only in melanoma biopsies, although the proto-oncogene is transcribed at low levels in several nontumorous tissues, like skin, gills, and brain (Adam *et al.*, 1991).[3] To decide whether the failure to detect the protein product is due to the limited sensitivity of the immunoblotting technique or to some posttranscriptional regulation, further experiments are required. For the EGF receptor,

[3] N. Dimitrijevic, personal communication.

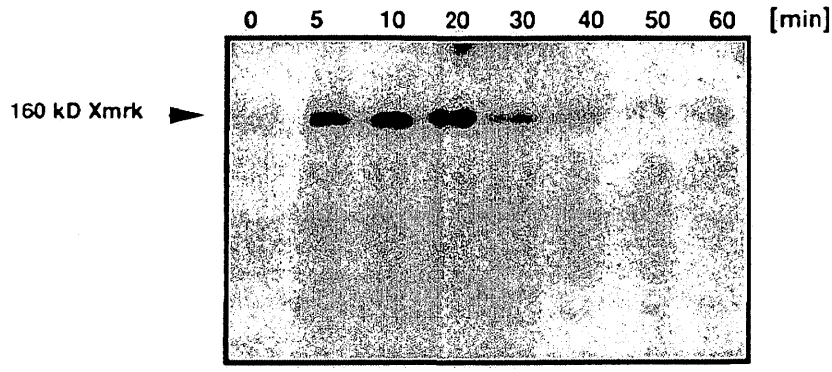

FIG. 9. **Time course of receptor autophosphorylation.** BHK cells stably expressing Xmrk were treated with conditioned medium from the PSM cells for different time periods prior to lysis. The cells were lysed in sample buffer, and the proteins were separated on a SDS-polyacrylamide gel. The gel was blotted and immunoprobed with the mAb αPY.

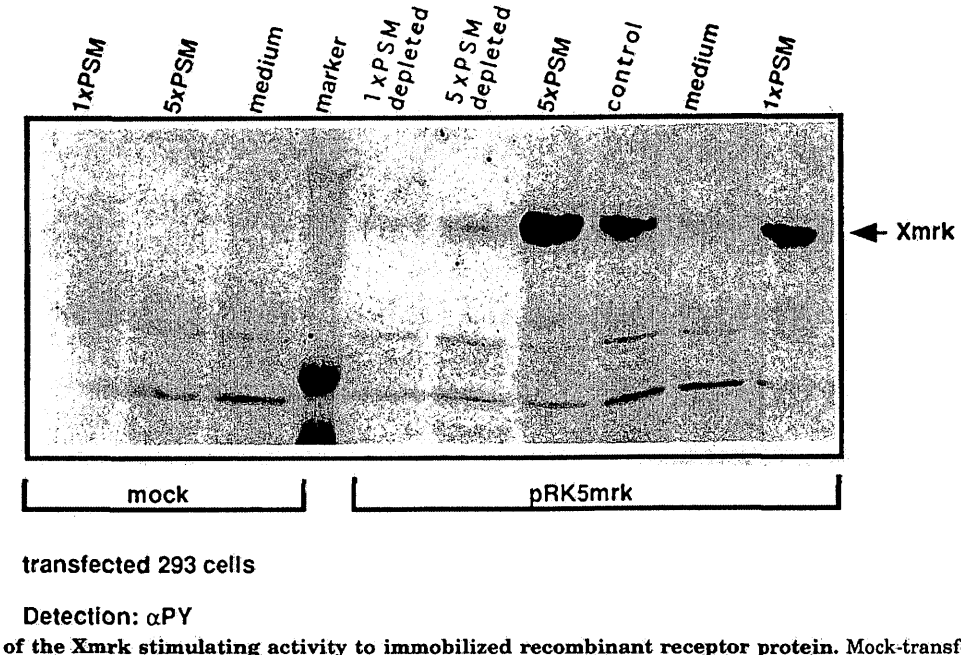

FIG. 10. **Binding of the Xmrk stimulating activity to immobilized recombinant receptor protein.** Mock-transfected or pRK5 Xmrk-transfected 293 cells were stimulated either with ultrafiltration concentrated (5 × PSM) or unconcentrated (1 × PSM) conditioned PSM medium. Where indicated, the conditioned medium was depleted through a ligand-affinity matrix. As controls, unconditioned medium (*medium*) and 1 × PSM that was depleted over a glutathione-Sepharose column where only the GST-PLCγ fusion protein, but not the receptor, had been adsorbed to (*control*) were used. The proteins were separated by SDS-PAGE, blotted, and detected with the αPY antibody.

it was shown that stimulation with EGF leads to higher receptor mRNA levels in the cells and that this up-regulation of the transcript is a posttranscriptional effect (Clark *et al.*, 1985; Earp *et al.*, 1986) and might be due to higher mRNA stability in stimulated cells (Jinno *et al.*, 1988). With regard of these results, one could speculate that an efficient translation of the *Xmrk* gene can only occur in tissues expressing a suitable ligand.

To detect the presence of a putative ligand, we measured the activation of the receptor by examining the state of autophosphorylation. In a previous study, we could show that there is a basal level of receptor autophosphorylation when the *Xmrk* gene is expressed in heterologous systems, such as transient expression in human embryonic kidney fibroblasts (293 cells) and stable expression in BHK cells. We could demonstrate that this basal activity is due to an activating mutation in the extracellular or transmembrane part of the receptor (Wittbrodt *et al.*, 1992). However, this activating mutation in the native receptor is not sufficient to lead to a transformation of 3T3 cells in the focus formation assay (Wittbrodt *et al.*, 1992). In the same system, a hEGFR/Xmrk chimera in the presence of EGF is a highly potent oncoprotein. To explain the high transforming potential of Xmrk in the Xiphophorus melanoma system, another mechanism of activation must be postulated. In the melanoma-derived PSM cell line of Xiphophorus, the receptor was determined to be highly autophosphorylated, suggesting that autocrine receptor stimulation due to production of the physiological ligand may be such an additional mechanism. We demonstrated that the PSM cells secrete a factor into the cell culture medium that can significantly enhance the basal level of activation in the heterologous assay systems. This factor not only induces receptor phosphorylation, but also receptor down-regulation as was seen after prolonged incubation of expressing cells with the conditioned medium. Furthermore, the stimulating activity could be depleted very specifically and efficiently by incubation with the immobilized recombinant receptor. Taken together, these data strongly suggest that the PSM cells produce the physiological ligand for the Xmrk receptor and grow autonomously via an autocrine pathway. Such autocrine systems have been identified in normal as well as transformed cells (reviewed in Sporn and Roberts (1985); Heldin *et al.*, 1987). The expression of the growth factors in transformed cells may be caused by different mechanisms. In an indirect autocrine pathway, the expression of a growth factor is triggered by

intracellular oncogenes at the transcriptional level. This was shown for the $p^{21K\text{-}ras}$- or $p37^{mos}$-dependent production of transforming growth factor-α in Mo-MSV and Ki-MSV transformed cells (Ozanne *et al.*, 1980; Kaplan *et al.*, 1982). It might be that the overexpression together with the basal activity of the Xmrk receptor leads to a comparable up-regulation of the production of the Xmrk ligand in the PSM cells. On the other hand, transformation of PDGF-receptor-expressing cells by the *sis* oncogene, which encodes a PDGF-like molecule (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983) is an example of a direct autocrine pathway. This autocrine receptor activation is responsible for cell transformation of simian sarcoma virus-infected cells as was shown by the antagonistic action of PDGF antibodies (Johnsson *et al.*, 1985). But the expression of growth factors in tumor cells may also reflect a normal function of the progenitor cell. Many normal cell types are known to produce growth factors in an autocrine manner, dependent on their stage of differentiation and activation. Examples include transforming growth factor-α in murine embryonal cells (Lee *et al.*, 1985) and PDGF in endothelial cells and activated macrophages (Jaye *et al.*, 1985; Martinet *et al.*, 1986). It seems most likely that in the PSM cells the expression of the Xmrk-specific growth factor represents a certain developmental stage of the normal counterpart of the melanoma cells as none of the additional oncogenic conversions (like retrovirus infection) described so far in autocrine tumor cell systems has been found in this cell line.

Although in Xiphophorus melanoma formation, the transcriptional activation of a single gene, the *Xmrk* gene, is necessary and sufficient to initiate neoplastic transformation of pigment cells, there are additional factors which must be present for the development of the malignant phenotype. The ligand of Xmrk is one of such additional factors. Identification of this protein will help to elucidate the multistep nature of the Xmrk-initiated tumorigenesis in Xiphophorus. The PSM cells can serve as a source for the purification of this new growth factor. The analysis of its expression pattern and biochemical functions is expected to contribute to an understanding of autocrine activation processes in tumor formation.

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