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The Xmrk receptor tyrosine kinase is activated in *Xiphophorus* malignant melanoma

Joachim Wittbrodt[1], Reiner Lammers[2], Barbara Malitschek, Axel Ullrich[2] and Manfred Schartl[3]

Max-Planck Institute for Biochemistry, Genecenter, Am Klopferspitz 18a, D-8033 Martinsried, [1]Biocenter of the University of Basel, Department of Cell Biology, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and [2]Max-Planck Institute for Biochemistry, Am Klopferspitz 18a, D-8033 Martinsried, Germany

[3]Corresponding author (present address: Biocenter, Department of Physiological Chemistry I, University of Würzburg, Am Hubland, D-8700 Würzburg, Germany)

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X*mrk* encodes a putative transmembrane glycoprotein of the tyrosine kinase family and is a melanoma-inducing gene in *Xiphophorus*. We attempted to investigate the biological function of the putative Xmrk receptor by characterizing its signalling properties. Since a potential ligand for Xmrk has not yet been identified, it has been difficult to analyse the biochemical properties and biological function of this cell surface protein. In an approach towards such analyses, the Xmrk extracellular domain was replaced by the closely related ligand-binding domain sequences of the human epidermal growth factor receptor (HER) and the ligand-induced activity of the chimeric HER-Xmrk protein was examined. We show that the Xmrk protein is a functional receptor tyrosine kinase, is highly active in malignant melanoma and displays a constitutive autophosphorylation activity possibly due to an activating mutation in its extracellular or transmembrane domain. In the focus formation assay the HER-Xmrk chimera is a potent transforming protein equivalent to other tyrosine kinase oncoproteins.
Key words: chimeric RTKs/melanoma/RTK/*Xiphophorus*

Introduction

In multicellular organisms, cell surface receptors play a pivotal role in the communication process of a cell with its environment. Receptor tyrosine kinases (RTKs) constitute a group of such molecules that possess a specific intracellular enzymatic activity, which upon binding of a ligand, initiates a signal transduction process that results in a specific cellular response. Questions of major interest center around the elucidation of the molecular components of such systems, as well as the biological role that each of the multiple ligand-receptor systems plays. Biochemical analysis is complicated by the pleiotropic character and complexity of the receptor-activated signal transduction pathways. For functional analysis, several animal systems provide promising tools where due to a genetic defect affecting the ligand-stimulated signal transduction pathway, a specific mutant phenotype is evoked. Several such systems exist for studies of the physiological functions of RTKs, e.g. the *W* and *steel* loci of mice, which define functions of the c-Kit RTK and its corresponding ligand (Geissler *et al.*, 1988; Williams *et al.*, 1990), the vulval induction locus (let-23) of *Coenorhabditis elegans* (Aroian *et al.*, 1990) and the *faint little ball* and *torpedo* mutants of *Drosophila* (Price *et al.*, 1989; Schrejter and Shilo, 1989), all of which affect the EGF receptor, the *sevenless* gene encoding a unique *Drosophila* RTK that affects the eye development (for review see Basler and Hafen, 1988) and the early developmental RTK *torso*, which is a key component of the *Drosophila* terminal organizer system (Sprenger *et al.*, 1989). In addition to their physiological function, several RTKs appear as crucial effectors in the process of neoplastic transformation.

The melanoma system of the swordtail fish *Xiphophorus* (for review see Schartl and Adam, 1992), offers a unique vertebrate model for the analysis of the physiological function of RTKs in tumorigenesis. X*mrk* has been identified as the melanoma-inducing oncogene in *Xiphophorus* (Wittbrodt *et al.*, 1989). In addition to the oncogenic X*mrk*, a second homologous copy of the gene representing the proto-oncogene has been found in the genome of *Xiphophorus*. The nucleotide sequence of the oncogenic X*mrk* was obtained from a cDNA isolated from melanoma cells. Analysis of its predicted primary structure revealed that it contains all of the major structural features of a cell surface receptor with tyrosine kinase activity. It bears the greatest similarity to members of RTK subclass I in its extracellular, juxtamembrane and kinase domains (Yarden and Ullrich 1988; Ullrich and Schlessinger, 1990). The predicted X*mrk* gene product, like all subclass I RTKs, contains an uninterrupted cytoplasmic tyrosine kinase domain that is connected via hydrophobic transmembrane sequences with two cysteine-rich repeat sequences in the extracellular ligand-binding domain. While the EGF receptor is known to bind EGF, transforming growth factor α (TGFα) and the vaccinia virus growth factor, the ligand of the X*mrk* gene product is still unknown, and its normal biological role is not yet clear. An involvement of the Xmrk receptor in cellular growth control mechanisms is suggested by the fact that uncontrolled expression of the X*mrk* gene leads to formation of malignant melanoma in *Xiphophorus* hybrids (Wittbrodt *et al.*, 1989; Adam *et al.*, 1991).

To understand the function of the Xmrk protein for the process of neoplastic transformation of pigment cells, its biochemical and biological properties have to be characterized. To study this prior to the identification of its specific ligand, we have constructed a chimeric receptor. Using the related, but distinct EGF receptor (human epidermal growth factor receptor, HER) ligand-binding and transmembrane domain fused to the cytoplasmic portions of Xmrk, we were able to investigate the signalling function of the Xmrk cytoplasmic domain by activation with EGF. We demonstrate that Xmrk has the potential to generate a normal signal when stimulated by a ligand and shows

constitutive autophosphorylation activity in heterologous expression systems. It is highly active and the most abundant phosphotyrosine protein in malignant melanoma of *Xiphophorus*. The HER−Xmrk chimera is a potent oncoprotein that readily leads to cell transformation upon ligand binding.

Results

Expression of Xmrk and of HER−Xmrk chimeric receptor in heterologous and homologous cells

To express the Xmrk protein transiently, the coding region of the Xmrk cDNA was cloned into the eukaryotic expression vector pRK5, generating the pRK5mrk construct (Figure 1). This expression construct was transfected to 293 cells (human embryonic kidney fibroblasts).

The antiserum directed against the RK-2 peptide, derived from the C-terminus of the HER (Kris *et al.*, 1985), was expected to cross-react with the Xmrk protein, as it shares 10 out of 12 amino acids in the corresponding C-terminal region (Figure 2a). The RK-2 antiserum specifically precipitated a 160 000 polypeptide (or a 160−150 kDa doublet) from pRK5mrk transfected 293 cells, but not from mock transfected cells (Figure 2b). A protein of the same size was detected with the RK-2 antiserum in the lysate of PSM cells (Platy-Swordtail melanoma cells), which have been shown to express abundantly Xmrk at the RNA level (Mäueler *et al.*, 1988; Wittbrodt *et al.*, 1989). No such protein was precipitated from the non-tumorous A2 embryonal cell line. The difference between the predicted MW of the Xmrk protein (127 kDa) and the apparent MW of 160 kDa determined from SDS gels, suggests a post-translational modification. As the conceptual translation of the cDNA predicted 13 N-glycosylation sites in the extracellular domain glycosylation is the most probable modification. The identical size of the proteins detected in Xmrk-expressing PSM and 293 cells suggests that the fish Xmrk protein is correctly expressed and processed in the mammalian 293 cell system.

To examine the biochemical properties and biological signalling potential of the putative growth factor receptor Xmrk prior to the identification of its cognate ligand, we replaced its extracellular and transmembrane domain with the ligand-binding domain of HER. The fusion of the corresponding cDNAs was carried out at the common *Nar*I site (HER bp 2195, Ullrich *et al.*, 1984; Xmrk bp 2028, Wittbrodt *et al.*, 1989) at the intracellular border of the HER and Xmrk transmembrane sequence (see Figure 1; Materials and methods). The hybrid cDNA was inserted into the mammalian expression vector pRK5 to generate the pRK5HER−Xmrk expression construct.

By DNA transfection of human 293 cells were generated transiently expressing either the HER−Xmrk chimera or wild type HER and Xmrk as controls.

Immunoprecipitates from lysates of [^{35}S]methionine-labelled cells using either mAb 108.1, a monoclonal antibody directed against extracellular domain III of HER (Lax *et al.*, 1989) or the polyclonal RK-2 antiserum, both detected a protein of 165 kDa. This demonstrated that the chimeric molecule synthesized in transfected 293 cells contained N-terminal HER sequences as well as C-terminal structures that cross-react with RK2, suggesting a correct translation of the HER−Xmrk chimera (Figure 3). The chimeric HER−Xmrk protein (calculated MW 127 600 Da) migrated in SDS gels with an estimated MW of 165 kDa, suggesting correct glycosylation of the EGF receptor extracellular domain of HER−Xmrk.

No difference was found in the 293 cell system with the mAb 108.1 in the expression levels of the HER−Xmrk chimeric protein and the wild type HER RTK when

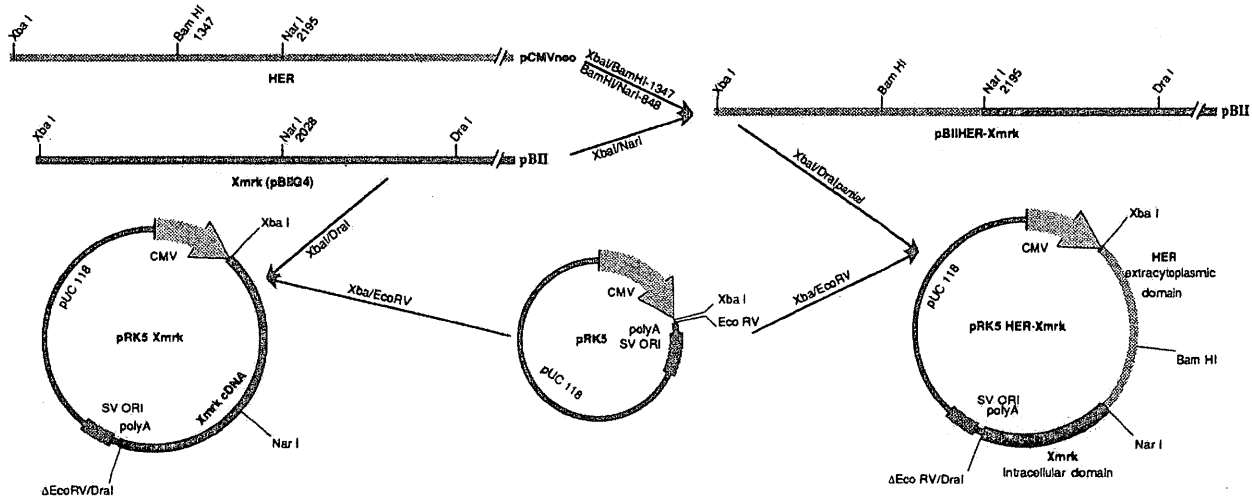

Fig. 1. Construction and physical maps of expression constructs. To obtain an expression construct for *Xmrk*, the entire coding sequence of *Xmrk* was removed from the plasmid pBII G4 by digestion with *Xba*I and *Dra*I, and ligation to the expression vector pRK5, which had been digested with *Xba*I and *Eco*RV. This resulted in the expression construct pRK5 Xmrk. For the cloning of a chimeric expression construct, the plasmid pBII G4 was digested with *Xba*I and *Nar*I to remove the sequences encoding the extracytoplasmic portion of the putative protein. The expression plasmid CMVneoHER, which contained the entire human EGF receptor gene under the control of the CMV enhancer, was digested with *Nar*I and *Bam*HI, and *Xba*I and *Bam*HI. A 1347 bp *Xba*I−*Bam*HI fragment and a 848 bp *Bam*HI−*Nar*I fragment, containing the extracellular and transmembrane domain-encoding sequences of HERI isolated from the plasmid CMVneoHER, was added to the pBII G4 *Xba*I−*Nar*I fragment and joined to yield the pBII HER−Xmrk plasmid in a three-factor ligation. The resulting plasmid contained the extracellular and transmembrane domain encoding sequences of HERI fused to the cytoplasmic domain encoding sequences of *Xmrk*. This plasmid was cut with *Xba*I and partially digested with *Dra*I. A 3.6 kb *Xba*I−*Dra*I fragment, containing the entire coding sequence, was ligated into the expression vector pRK5 which had been digested with *Xba*I and *Eco*RV to yield in the expression construct pRK5 HER−Xmrk. The precision of the ligations was confirmed by sequence analysis.

comparing immunoprecipitates from metabolically labelled transfected 293 cells (data not shown).

In vitro and in vivo autophosphorylation

To examine the functional integrity of the HER−Xmrk chimera, we tested the ability of the EGF receptor ligand-binding domain to activate the kinase of the Xmrk cytoplasmic portion upon ligand-binding *in vitro*, thus resulting in receptor phosphorylation on tyrosine residues. 293 cells expressing HER or HER−Xmrk were stimulated with EGF. Cells were lysed and the crude membrane extract was immunoprecipitated with mAb 108.1. The *in vitro* kinase assay demonstrated that like native HER, the chimeric HER−Xmrk protein displays autophosphorylation that can be stimulated by EGF *in vitro* (Figure 4).

The autophosphorylation of Xmrk and HER−Xmrk was further investigated by experiments with living cells utilizing phosphotyrosine antibodies. 293 cells that were transiently expressing Xmrk or HER−Xmrk were metabolically labelled with [^{35}S]methionine and exposed to EGF. The reactions were stopped after 10 min by cell lysis with Triton lysis buffer. Samples were immunoprecipitated using an antibody directed against phosphotyrosine and separated by SDS gel electrophoresis. Tyrosine-phosphorylated proteins were then detected by autoradiography.

In cells that were expressing HER−Xmrk, a band of 165 kDa was found to be tyrosine-phosphorylated only after EGF stimulation. In the absence of EGF, no tyrosine-phosphorylated protein could be detected.

To analyse the chimeric receptor in fish cells, which provide a homologous system for the Xmrk kinase, A2 cells transiently expressing HER−Xmrk were treated with EGF and lysed. Compared with 293 cells, which were transfected in a parallel experiment, A2 cells expressed HER−Xmrk at much lower levels (data not shown). To prove conclusively that the tyrosine-phosphorylated proteins detected represented autophosphorylated HER−Xmrk, the samples were immunoprecipitated using the mAb 108.1, separated on SDS gels, Western blotted and immunoprobed with an antibody directed against phosphotyrosine. As shown in Figure 6, tyrosine phosphorylation of the chimeric HER−Xmrk protein was strongly induced by EGF in HER−Xmrk-expressing cells, whereas the chimeric RTK was quiescent in unstimulated cells. This was equally true for mammalian (293) and fish (A2) cells expressing the chimeric RTK.

Due to the structural similarity of the Xmrk and HER extracellular domains, it was reasonable to investigate whether other ligands known to stimulate HER kinase activity also interact with Xmrk. Metabolically labelled 293 cells that were expressing X*mrk* were treated with EGF, lysed, immunoprecipitated using an antibody directed against phosphotyrosine and separated by SDS gel electrophoresis. Tyrosine-phosphorylated proteins were detected by autoradiography. EGF as well as TGFα (data not shown)

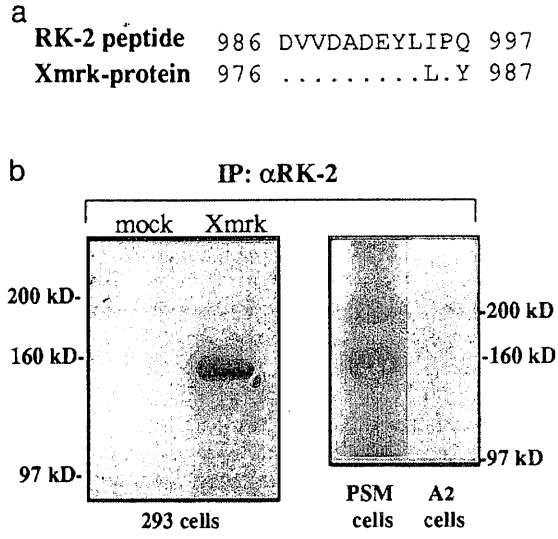

Fig. 2. Detection of the Xmrk receptor protein in Human embryonic kidney fibroblasts (293) transiently expressing Xmrk and non-transfected fish melanoma cells (PSM). **a.** Comparison of the RK-2 peptide and the corresponding region of the Xmrk protein. **b.** Cells were labelled with [^{35}S]methionine overnight, lysed and immunoprecipitated as described in Materials and methods. Lysates were immunoprecipitated with αRK-2 antiserum. Mock-transfected 293 cells and fish A2 cells not expressing Xmrk were used as controls. Molecular weight markers are indicated in kDa.

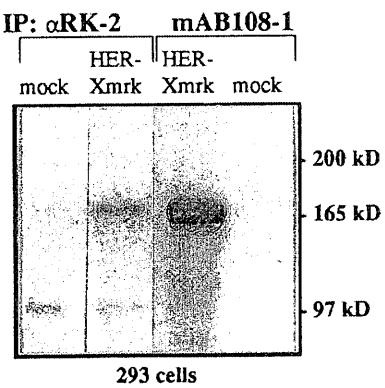

Fig. 3. Detection of chimeric HER−Xmrk receptors. 293 cells transfected with the chimeric HER−Xmrk expression construct were labelled with [^{35}S]methionine overnight, lysed and immunoprecipitated with the indicated antisera as described in Materials and methods. Mock-transfected 293 cells were used as controls. Molecular weight markers are indicated in kDa.

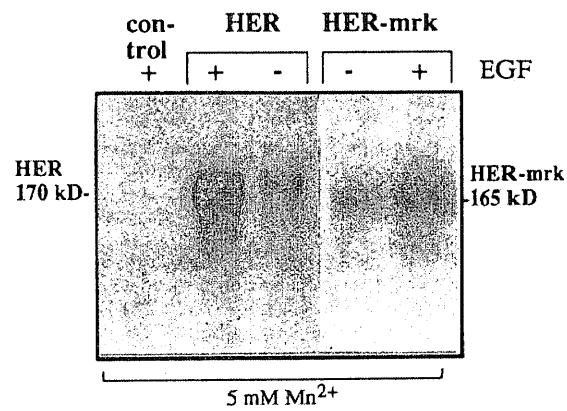

Fig. 4. Receptor autophosphorylation *in vitro*. Equal numbers of 293 cells transiently expressing HER and HER−Xmrk were incubated in the presence (+) or absence (−) of 50 ng/ml EGF, lysed and the lysates immunoprecipitated with the monoclonal antibody mAb 108.1. *In vitro* kinase reactions were carried out as described in Materials and Methods. Mock-transfected cells were used as control. Molecular weight markers are indicated in kDa.

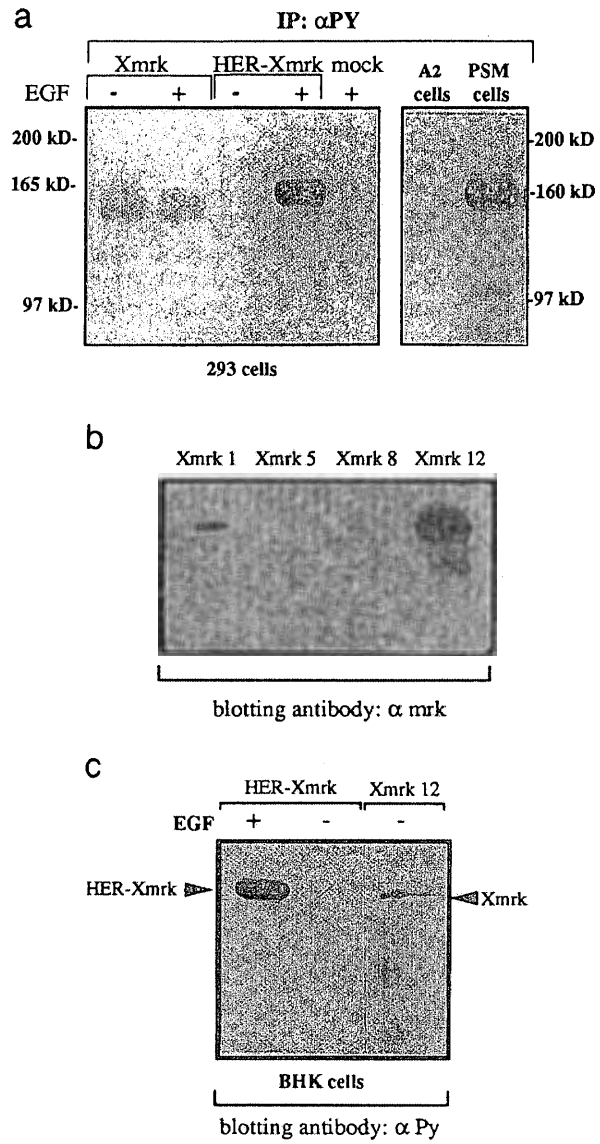

Fig. 5. Receptor autophosphorylation in living cells. (a) Equal numbers of 293 cells transiently expressing Xmrk and HER−Xmrk as well as A2 and PSM cells were metabolically labelled with [^{35}S]methionine overnight, incubated in the presence (+) or absence (−) of 50 ng/ml EGF, lysed and the lysates immunoprecipitated with a monoclonal antibody directed against phosphotyrosine. Mock-transfected and A2 cells were used as control. Molecular weight markers are indicated in kDa. (b) Western blot analysis of BHK cells expressing Xmrk. Equal numbers of Xmrk-expressing BHK cells were lysed, the proteins were separated by SDS−PAGE, the gels were blotted and immunoprobed with the αXmrk serum and detected with the ECL chemiluminescence system (Amersham). (c) Analysis of tyrosine phosphorylation in stably expressing BHK cells. HER−Xmrk and Xmrk-expressing cells were incubated in the presence (+) or absence (−) of 50 ng/ml EGF prior to lysis. Lysates were separated on SDS gels, blotted and the blots were immunoprobed with the αPY antiserum and detected with the ECL chemiluminescence system (Amersham).

were found to have no influence on the phosphorylation level of the 160/150 000 double band representing Xmrk (Figure 5a).

In contrast to the HER−Xmrk chimeric protein, in which the Xmrk kinase is quiescent unless activated with EGF, there was a significant basal activity of the Xmrk kinase in 293 cells transiently expressing Xmrk (Figure 5a).

To further investigate the phenomenon of Xmrk autophosphorylation found in transiently expressing 293 cells, the *Xmrk* gene and the HER−Xmrk chimera were transfected into BHK cells using the CVN vector to obtain stably expressing lines. For the chimera two stable lines (HM9 and HM8) were generated expressing the receptor at low and high levels, respectively. Similar to the situation in 293 cells the chimeric receptor was not tyrosine-phosphorylated in the absence of ligand (Figure 5c). Addition of EGF led to a strong autophosphorylation. Five lines (Xmrk1, 7, 8, 11 and 12) expressed the native Xmrk receptor stably at different levels (Figure 5b and data not shown). In clone Xmrk12 giving the highest expression comparable to the expression levels of HER−Xmrk in HM8, a constitutive autophorphorylation of Xmrk was readily detected (Figure 5c).

To analyse Xmrk kinase activity *in vivo*, PSM fish melanoma cells and various normal and tumorous tissues of *Xiphophorus* were used. The immunoprecipitation of a Triton-X100 lysate of metabolically-labelled PSM melanoma cells with an αPY antibody revealed a strongly tyrosine-phosphorylated band of 160 kDa corresponding to the 160 kDa Xmrk protein detected with the RK-2 antiserum (Figures 1 and 5). Biopsies of malignant melanomas of *maculatus−Xiphophorus helleri* hybrids, as well as of normal tissues, were lysed and immunoprecipitated with an antiserum raised against Xmrk protein expressed in recombinant baculovirus-infected SF9 insect cells (Malitschek *et al.*, in preparation). The precipitated proteins were separated on SDS gels, Western blotted, and immunoprobed with αPY antibodies.

Xmrk that was precipitated from melanoma biopsies was found to be strongly phosphorylated on tyrosine. Gills that were demonstrated to express the *Xmrk* proto-oncogene transcript (Wittbrodt *et al.*, 1989) and *Xiphophorus* brain lysates did not to contain any αXmrk-precipitable tyrosine-phosphorylated protein (Figure 6).

In summary, the tyrosine kinase of the chimeric HER−Xmrk protein can be activated by human EGF, but is inactive in unstimulated cells. The Xmrk protein was found to be phosphorylated on tyrosine in *Xmrk*-expressing heterologous cells. In fish melanoma cells and in melanoma biopsies, the Xmrk tyrosine kinase is strongly phosphorylated on tyrosine.

Substrate phosphorylation

Upon activation with their ligands, receptors of the tyrosine kinase family induce similar primary responses within cells (for review see Ullrich and Schlessinger, 1990). RTKs act by phosphorylating substrate proteins on tyrosine and thereby activate their substrates. To investigate the substrate phosphorylation of the Xmrk kinase upon activation with EGF in the HER−Xmrk chimera and compare it with the substrate phosphorylation of the HER, we transfected 293 cells with HER or HER−Xmrk expression constructs and stimulated cells with EGF. Triton lysates were directly separated on SDS gels, Western blotted and the blots were immunoprobed with αPY antibodies.

In cells that were expressing HER−Xmrk, a tyrosine-phosphorylated protein of 140 kDa could be detected at abundant levels after EGF stimulation (Figure 7). In unstimulated cells expressing HER−Xmrk this 140 kDa phosphoprotein is present at low levels, whereas in HER

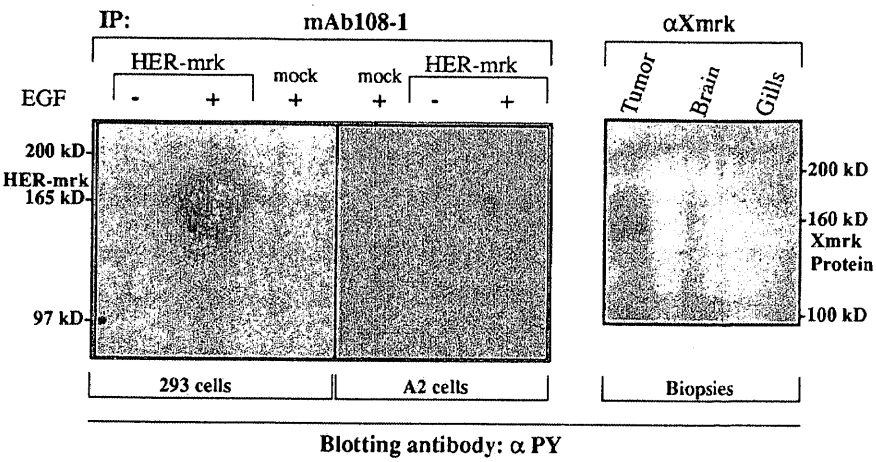

Fig. 6. Western blot analysis of the autophosphorylation of the Xmrk kinase domain in heterologous and homologous cells and tissues. Human 293 cells and fish A2 cells transiently expressing HER−Xmrk were incubated in the presence (+) or absence (−) of 50 ng/ml EGF. Equal numbers of cells were lysed and the lysates immunoprecipitated with a monoclonal antibody directed against the second cysteine rich domain of HER. Immunoprecipitates were separated on SDS gels, Western blotted and the blots immunoprobed with antibodies directed against phosphotyrosine. Tyrosyl phosphorylated proteins were detected by autoradiography after incubation with [^{125}I]protein A. (Mock-transfected and A2 cells were used as control.) Triton lysates of indicated tissues were immunoprecipitated with a polyclonal antiserum directed against baculovirus expressed Xmrk. Immunoprecipitates were separated on SDS gels, Western blotted and immunoprobed with antibodies directed against phosphotyrosine. Tyrosine-phosphorylated proteins were detected by chemoluminescence with an HRP coupled second anti-rabbit antibody. Molecular weight markers are indicated in kDa.

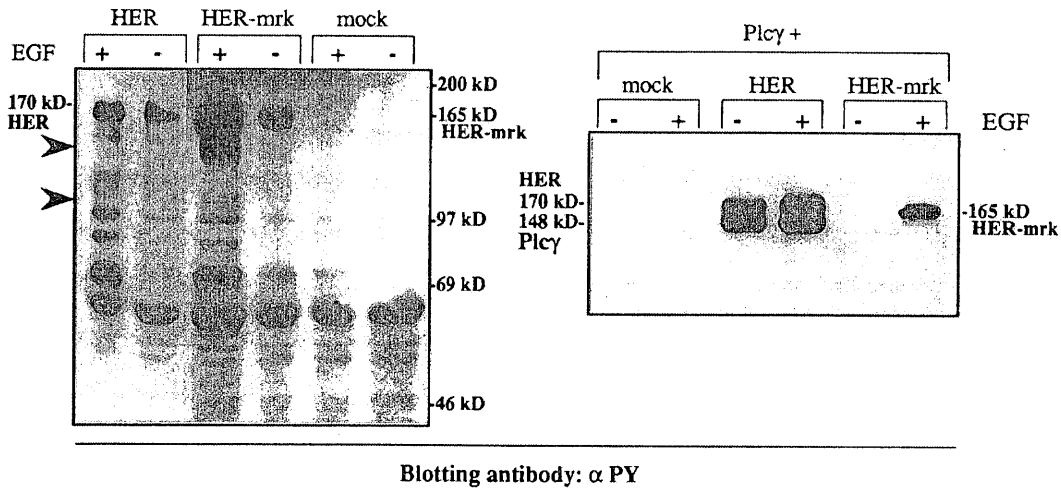

Fig. 7. EGF-stimulated *in vivo* phosphorylation of receptor substrates. **a.** Confluent monolayers of cells transiently expressing HER and HER−Xmrk as well as mock transfected cells were incubated in the presence (+) or absence (−) of 50 ng/ml EGF. Following stimulation, equal amounts of cells were lysed and lysates were directly separated on SDS gels. After Western blotting, membranes were immunoprobed with an antibody directed against phosphotyrosine and tyrosine-phosphorylated proteins detected by chemoluminescence after incubation with an HRP coupled second anti-mouse antibody. (Mock-transfected 293 cells were used as control.) **b.** Equal numbers of cells cotansfected with PLCγ and HER or HER−Xmrk or control DNA were lysed and the lysates separated on SDS gels, Western blotted and the blots immunoprobed with antibodies directed against phosphotyrosine. Tyrosine-phosphorylated proteins were detected as above. Molecular weight markers indicated are in kDa.

and mock controls it is barely detectable. A second less prominent candidate substrate of the Xmrk kinase in 293 cells had an approximate MW of 105 kDa. A 72 kDa protein was found to be tyrosine-phosphorylated in HER- and HER−Xmrk-expressing cells after stimulation with EGF (Figure 7).

A prominent substrate of HER is PLCγ, which becomes tyrosine phosphorylated (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989) and thereby activated (Nishibe *et al.*, 1990) in HER-expressing cells upon stimulation with EGF. C-terminal autophosphorylation sites have to be phosphorylated for a receptor/PLCγ interaction (Margolis *et al.*, 1990). The high similarity of HER and Xmrk, and the conservation of the autophosphorylation sites suggested that PLCγ may also be a substrate for the Xmrk kinase. To analyse the interaction of the Xmrk fish tyrosine kinase and human PLCγ, 293 cells were cotransfected with PLCγ and HER−Xmrk expression constructs and treated as described above. Cells coexpressing HER and PLCγ exhibited strong phosphorylation of the 170 kDa receptor protein and the 148 kDa PLCγ even in the absence of EGF. The tyrosine phosphorylation of the HER−Xmrk chimera was stimulated by EGF, but only a very faint band of 148 kDa representing the tyrosine-phosphorylated PLCγ was

detected in the EGF-stimulated cells coexpressing HER−Xmrk and PLCγ (Figure 7b).

Several potential substrates of the Xmrk tyrosine kinase, different from substrates of HER appeared to be tyrosine-phosphorylated after EGF treatment of HER−Xmrk-expressing 293 cells. However so far, human PLCγ cannot be classified as a major substrate of the fish Xmrk kinase in the coexpression assay. Further experiments are needed to clarify this point.

Transforming ability of the HER-Xmrk chimera

To investigate whether the HER-Xmrk chimera mediates growth signals in a heterologous system, NIH 3T3 cells were infected with replication defective retroviruses that carry the cDNAs coding for Xmrk or HER-Xmrk. No foci were obtained in the *Xmrk* infected cells neither in the presence nor absence of EGF. A low level of foci formation was observed in the HER-Xmrk expressing cells without addition of exogenous EGF (1.2 foci/10^3 viruses). This can be explained by unavoidable remnant traces of EGF in the tissue culture medium. In the presence of EGF the number of foci was dramatically increased to one focus/virus.

Discussion

We have constructed a receptor chimera to initiate the characterization of the biochemical and biological functions of Xmrk and thereby its yet unidentified ligand. This approach has previously been successful in creating functional insulin−IGF1 receptors, insulin−EGF receptor chimera and HER1−HER2 hybrids (Riedel *et al.*, 1986; Lammers *et al.*, 1989; Lee *et al.*, 1989, Lehväslaiho *et al.*, 1989). Using the EGF receptor ligand-binding domain fused to the Xmrk cytoplasmic domain, we were able to investigate various aspects of biological signalling activities normally induced through the Xmrk molecule by its currently unknown ligand. The HER−Xmrk chimera was correctly synthesized, processed and transported to the cell surface.

Ligand-induced autophosphorylation is thought to be a crucial step in the process of receptor-mediated signalling. Defects in this process either lead to a loss of biological activity or constitutive activation or loss of control. It has been shown that ligand binding to a receptor chimera results in activation of the heterologous receptor kinase and autophosphorylation *in vitro* and in living cells (Riedel *et al.*, 1986; Lammers *et al.*, 1989; Lee *et al.*, 1989; Lehväslaiho *et al.*, 1989). This process is also functional in the HER−Xmrk receptor hybrid. *In vitro* as well as in intact cells, the stimulation of the HER−Xmrk chimera with EGF led to the induction of the intracellular tyrosine kinase, as measured by the degree of receptor autophosphorylation. This confirms that a heterologous cytoplasmic domain does not disturb the formation of an authentic binding site conformation by EGF receptor extracellular sequences, even between domains that have undergone as much as 900 million years of independent evolutions.

In addition to retaining full kinase activity, HER−Xmrk was also able to generate an apparently Xmrk-specific signal that resulted in phosphorylation of cellular protein substrates. This demonstrates that despite the high similarity of HER and Xmrk in their primary structure, receptor-specific signals may be generated within a single cell type by specific phosphorylation of different substrates. These specific substrates offer the opportunity to dissect the kinase domain in chimeric RTKs and thereby define the subdomains that confer to substrate specificity, in an approach similar to the characterization of the ligand-binding domain (Lax *et al.*, 1989).

Despite these differences in substrate specificity the high transforming activity of the HER−Xmrk chimera in NIH 3T3 cells dramatically shows the conservation of at least some components of the signalling pathways. The signal transducing machinery of the mammalian cells is obviously perfectly able to interact structurally and functionally with the fish receptor intracellular domain and elicit the same response as if it would be activated by a homologous molecule.

The native Xmrk protein is abundantly autophosphorylated in fish melanoma cells, where it is clearly the transforming oncogene. To explain the autophosphorylation of the Xmrk protein in transiently expressing 293 cells and stably expressing BHK cells, several arguments have to be considered. Firstly, this autophosphorylation may be a consequence of the high expression. However, the HER−Xmrk chimera, which is expressed under the control of the same promoter at similar levels, did not show detectable autophosphorylation without its ligand. In addition, in stable lines the expression level is much lower than in transient expression systems. The level of constitutive autophosphorylation of Xmrk was considerably lower than the autophosphorylation obtained after stimulation of HER or HER−Xmrk with the cognate ligand. It was detectable only in that stable BHK line which expresses the highest level of Xmrk. Secondly, the heterologous cells could produce a stimulatory factor for Xmrk. This explanation does not seem to be very likely in the light of the large evolutionary distance of fish and humans, because even the chicken EGF receptor is not able to recognize mammalian EGF with sufficient affinity (Lax *et al.*, 1989). Thirdly, Xmrk might carry an activating mutation in its extracellular or transmembrane domain, which then would be responsible for autostimulation. Similar phenomena have been observed in carcinogen-induced tumours of rats, where the *neu* oncogene was found to bear an activating mutation in the transmembrane domain (Bargmann and Weinberg, 1988; Stern *et al.*, 1988) and in v-*fms*, where point mutations in the extracytoplasmic portion led to an intracellular activation of the kinase (Roussel et al., 1988; Woolford *et al.*, 1988).

In the focus formation assay the native Xmrk RTK did not show any transforming ability. This may indicate that a low level of constitutive tyrosine phosphorylation at least in the heterologous cells is not sufficient to elicit a tumorigenic effect. From this it can be extrapolated that an activating mutation alone may not be the exclusive reason for the oncogenic function of Xmrk in neoplastic transformation of pigment cells. Further experiments are needed to clarify these points.

The complexity of Xmrk action is even higher if the *in vivo* situation of melanoma induction in *Xiphophorus* is considered. Interestingly, in the *Xiphophorus* melanoma system, the same gene that is responsible for tumour induction in hybrids is also present in the tumour-free parental feral animals. In addition to activation at the protein level, an alteration of the transcriptional control of the *Xmrk* gene by the crossing conditioned elimination of a tumor suppressor locus (Adam *et al.*, 1991) is crucial for neoplastic

transformation *in vivo*. Only unscheduled overexpression of the *Xmrk* oncogene in pigment cells of hybrid genotypes is the precondition to initiate the processes of Xmrk mediated tumorigenesis.

With this perspective, the *Xiphophorus* melanoma system provides not only a unique vertebrate model for the analysis of RTK function in melanoma formation, but also for suppression of RTK-mediated tumorigenesis.

Materials and methods

Antibodies

mAb 108.1 recognizes the extracellular domain of the human EGF receptor (Lax *et al.*, 1989), αPY was a monoclonal antibody directed against phosphotyrosine. Rabbit antiserum RK-2 recognizes residues 984–996 of the human EGF receptor (Kris *et al.*, 1985). Rabbit antiserum αXmrk was raised against Xmrk expressed in recombinant baculovirus infected SF9 insect cells (Malitschek *et al.*, in preparation).

Expression plasmids

The clone pBII G4, which contains the entire coding sequence of Xmrk, was generated by ligation of the extracellular and transmembrane domain containing clone p3-2E to the cytoplasmic domain containing clone p17-2 (Wittbrodt *et al.*, 1989) via the common *Eco*RI site (2153) in pBluescript II (Stratagene). The expression constructs for Xmrk and HER–Xmrk were cloned as described in Figure 1.

The construct CMVneoHER which contained the entire human EGF receptor gene under the control of the CMV enhancer, was used for HER expression. To produce the stably expressing BHK cell lines, the HER–Xmrk and Xmrk coding sequences were cloned in the CVN vector (Rosenthal *et al.*, 1986) by utilizing the *Xba*I and *Dra*I sites of the cDNAs and the *Xba*I and *Eco*RV sites in the vector.

For the production of ectotropic retroviruses, the cDNAs of Xmrk and HER–Xmrk were cloned into the retroviral expression vector pLXSN (Miller and Rosman, 1989). From the pRK constructs the cDNAs were isolated by a *Xba*I/*Dra*I partial digest for HER–Xmrk and *Hin*dIII/*Eco*RI partial digest for the Xmrk cDNA and ligated into *Hpa*I or *Hpa*I/*Eco*RI linearized vector.

The PLCγ expression construct for the coexpression experiments was obtained from J.Schlessinger.

Cell culture and transfections

293 human embryonic kidney fibroblasts were cultured in DMEM containing 10% fetal calf serum. Expression vectors were introduced into 293 cells by the method of Chen and Okayama (1987). 4 μg of supercoiled plasmid DNA were used per 3 cm dish. 15 h after transfection, the precipitate was removed from the cells by washing with PBS and cells were fed with fresh medium. Cell lysis was performed 36–40 h after transfection. A2 fish embryonal fibroblasts were cultured in F12 medium containing 10% fetal calf serum. Expression vectors were transfected into A2 cells as described earlier (Friedenreich and Schartl, 1990). BHK-21 cells were grown in DMEM/F12 mix supplemented with 10% fetal calf serum and antibiotics. DNA precipitate was prepared as above and added to 2×10^5 cells in a 3 cm dish. For selection, a puromycin resistance encoding plasmid (pSV2pac) was cotransfected in a molar ratio of 1:10. Transfected cells were selected in growth medium with 30 μg/ml of puromycin (Wirth *et al.*, 1988). Individual clones were picked and analyzed for expression.

The expression constructs pLXSN-Xmrk and pLXSN-HER-Xmrk were introduced into GP + E-86 cells by transfection which then produced ecotropic retrovirus. The virus for the focus formation assay was derived from cell culture supernatants of these GP + E-86 cells.

Focus formation assay

For the focus formation assay, 10^5 NIH 3T3 cells were seeded in 6 cm dishes and 24 h later, virus and 6 mg/ml polybrene were added. After 36 h the cells were trypsinized and reseeded into 10 cm dishes. The medium, containing 4% fetal calf serum, was changed every other day until foci appeared. For stimulation 10 ng/ml of EGF was added to the culture medium.

Metabolic labelling

For metabolic labelling of proteins in transiently expressing cells, cells were incubated 24 h after transfection with 1 ml of methionine-free medium containing 1% dialyzed fetal calf serum, 2 mM L-glutamine, antibiotics and 50 μCi/ml [^{35}S]methionine per 3 cm dish. The incubation was continued for 16 h.

Immunoprecipitation and Western blotting

To detect and characterize receptor proteins, cell monolayers were lysed in the dishes 36–40 h after transfection in 300 μl lysis buffer (20 mM HEPES pH 7.2, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride and 200 U/ml aprotinin). Dishes with lysis buffer were incubated on ice for 5 min, the supernatant was collected, incubated on ice for further 5 min, centrifuged at 14 000 *g* for 5 min and the supernatant was used for immunoprecipitation. The supernatant was diluted 1:3 with HNTG (20 mM HEPES pH 7.2, 150 mM NaCl, 10% glycerol and 0.1% Triton X-100) and incubated with 1–5 μl antiserum and 20 μl of protein A–Sepharose (1:1 slurry in HNTG buffer) for at least 2 h at 4°C. The immunoprecipitates were washed three times in 0.5 ml HNTG buffer. For analysis of tyrosine phosphorylation, cells were lysed and washed in lysis/HNTG buffer containing 100 mM NaF and 100 μM sodium orthovanadate. The pellet was then resuspended in 50 μl of 2×SDS gel sample buffer. Samples were boiled for 5 min, centrifuged and analysed on 7.5% SDS–polyacrylamide gels.

Gels containing proteins labelled with [^{35}S]methionine were fixed for 30 min in 10% acetic acid–25% isopropanol, incubated for 1 h in Amplify™, dried under vacuum at 80°C and exposed.

For total membrane extracts, the cells were lysed in 200 μl of lysis buffer. After centrifugation of the lysates, 50 μl of the supernatant were added to 50 μl of sample loading buffer, boiled for 30 min with the lids of the reaction tubes being open and electrophoresed on 7.5% SDS–polyacrylamide gels.

The proteins were transferred to nitrocellulose (Schleicher and Schuell) using standard Western blotting protocols.

Filters were blocked for 2×30 min with NET-G (150 mM NaCl, 5 mM EDTA, 0.05 % Triton X-100 and 0.25% gelatine) and treated 6 h with monoclonal antiphosphotyrosine antibody at 4°C, washed three times for 30 min in NET-G, incubated in [^{125}I]protein A for 2 h at room temperature, washed three times and exposed to X-ray film.

Non-radioactive detection (ECL-Amersham) was performed according to suppliers recommendations after blocking and incubation with first antibody.

In vitro phosphorylation assay

To determine receptor autophosphorylation activity, confluent 3 cm dishes of transfected cells were stimulated with EGF (100 ng/ml), lysed with lysis buffer as above and immunoprecipitated with mAb 108.1. The immunoprecipitate was resuspended in 10 μl HNTG-P containing 10 mM $MnCl_2$, 10 μCi of [γ-^{32}P]ATP were added to the immunoprecipitate and incubated 60 s on ice. 50 μl of hot sample buffer were added to terminate the reaction. Samples were then electrophoresed on 7.5% SDS–polyacrylamide gels. Dried gels were exposed to Kodak X-Omat AR film.

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