Ligand-independent Dimerization and Activation of the Oncogenic Xmrk Receptor by Two Mutations in the Extracellular Domain*

Ana Gómez‡, Claudia Wellbrock, Heidrun Gutbrod, Nicola Dimitrijevic§, and Manfred Schartl¶

From Physiological Chemistry I, Biocenter (Theodor Boveri Institute), University of Würzburg, Am Hubland, 97074 Würzburg, Germany

Overexpression of the oncogenic receptor tyrosine kinase ONC-Xmrk is the first step in the development of hereditary malignant melanoma in the fish Xiphophorus. However, overexpression of its proto-oncogene counterpart (INV-Xmrk) is not sufficient for the oncogenic function of the receptor. Compared with INV-Xmrk, the ONC-Xmrk receptor displays 14 amino acid changes, suggesting the presence of activating mutations. To identify such activating mutations, a series of chimeric and mutant receptors were studied. None of the mutations present in the intracellular domain was found to be involved in receptor activation. In the extracellular domain, we found two mutations responsible for activation of the receptor. One is the substitution of a conserved cysteine (C378S) involved in intramolecular disulfide bonding. The other is a glycine to arginine exchange (G359R) in subdomain III. Either mutation leads to constitutive dimer formation and thereby to activation of the ONC-Xmrk receptor. Besides, the presence of these mutations slows down the processing of the Xmrk receptor in the endoplasmic reticulum, which is apparent as an incomplete glycosylation.

Receptor tyrosine kinases (RTKs) are important components of the signaling network that controls cell growth and differentiation. Their enzymatic activity is tightly regulated in normal cells. After ligand binding and dimerization, they become activated and a cascade of phosphorylations is initiated inside the cells (1). Diverse mechanisms have been reported that can lead to the constitutive activation of these enzymes. These comprise overexpression, amplification, point mutations, truncations, and autocrine stimulation. The inappropriate constitutive activation of the RTKs results in an altered signaling inside the cell and is a widely documented process implicated in tumor formation (2, 3).

The hereditary melanoma of Xiphophorus fish is a well-established genetic model system for tumor development in which the overexpression of the RTK gene Xmrk (Xiphophorus melanoma receptor kinase) leads to melanoma formation (for review, see Ref. 4). Xmrk belongs to the epidermal growth factor receptor (EGFR) family, but it is an additional member, clearly distinct from the four receptors (HER1–4) already described in mammals (5). Two copies of the Xmrk gene have been found. One of them, INV-Xmrk, is a gene invariably present in all fish (6). It is ubiquitously expressed at low levels, and, although its physiological role is still unknown, it appears not to be involved in melanoma formation. It represents the proto-oncogenic form of Xmrk.

The second oncogenic copy, ONC-Xmrk, is only present in some species of Xiphophorus. It originated by an ancient gene duplication event from INV-Xmrk and it is under a different transcriptional control than the proto-oncogene. Only basal levels of expression are observed if the regulatory locus R is also present in the genome. This is the situation found in nonhybrid wild fish, which are generally tumor-free. In hybrids, due to crossing conditioned elimination of the R-containing chromosome (7), the system is deregulated and ONC-Xmrk is overexpressed (8). This leads to neoplastic transformation of pigment cells. A cell line (PSM) derived from Xiphophorus melanoma provides an in vitro system where ONC-Xmrk is also overexpressed. Here, as in melanoma in situ, the Xmrk receptor is highly activated, which is apparent as strong tyrosine autophosphorylation (9, 10).

The fact that the highly expressed ONC-Xmrk is constitutively autophosphorylated in melanoma cells pointed to overexpression, and thus high concentration of receptors, as one mechanism for activation. However, the ectopic overexpression of INV- and ONC-Xmrk in embryos of transgenic fish showed that exclusively those fish expressing ONC-Xmrk were developing tumors with high incidence, short latency periods, and a specific pattern of affected tissues, whereas only a basal rate of tumor induction appeared in the case of INV-Xmrk-expressing fish, comparable to the rate obtained with the expression of another, nonactivated receptor (6, 11). Besides that, INV-Xmrk was shown to be not phosphorylated when transiently expressed in human cells (HEK293), in contrast to the strong autophosphorylation of ONC-Xmrk. The different behavior of INV- and ONC-Xmrk clearly indicates that a mechanism additional to overexpression is instrumental in Xmrk activation (6).

Comparison of the amino acid sequences of the two versions of Xmrk revealed that the oncogene differs from the proto-oncogene in 14 residues, including some that are highly conserved.

Received for publication, July 24, 2000, and in revised form, October 17, 2000
Published, JBC Papers in Press, October 18, 2000, DOI 10.1074/jbc.M006574200

‡ Present address: Inst. de Acuicultura Torre la Sal, 12595 Castellón, Spain.
¶ Present address: Augsburg Hospital, Inst. for Pathology, D-86156 Augsburg, Germany.
§ Present address: Augsburg Hospital, Inst. for Pathology, D-86156 Augsburg, Germany.

* This work was supported by grants from Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 487; Regulatorische Membranproteine, Graduiertenkolleg: Zellwachstum) and Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; PAGE, polyacrylamide gel electrophoresis; endo H, endoglycosidase H; ER, endoplasmic reticulum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; ONC, oncogenic version of Xmrk; INV, Xmrk proto-oncogene product.

2 A. Gómez, unpublished data.
3 A. Gómez, M. Schartl, C. Winkler, and Y. Hong, unpublished data.
served in the EGFR family of RTKs and that are present in INV-Xmrk but substituted in ONC-Xmrk (6). This fact suggested that mutational alteration could be involved in the activation of Xmrk. Moreover, the high phosphorylation level shown by an ONC-INV chimeric receptor containing the extracellular domain of ONC-Xmrk and the intracellular domain of INV-Xmrk pointed to one or more of the mutations in the extracellular domain as implicated in the activation (6). However, this result did neither address a role of the intracellular mutations in the activation of Xmrk nor could it identify the extracellular oncogenic amino acid change.

To understand the mechanism of activation of ONC-Xmrk, we have analyzed the effect of different mutations. We show that two mutations in the extracellular region of the ONC-Xmrk receptor are responsible for activation. Both of them independently lead to the constitutive dimerization of the receptor by aberrant intermolecular disulfide bonding formation. Additionally, the presence of these mutations slows down the processing of Xmrk receptor in the endoplasmic reticulum (ER), which is apparent as an incomplete glycosylation.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—5E.2 (anti-Tyr(P)) is a mouse monoclonal antibody directed against phosphotyrosines (12). Anti-mrk is a polyclonal antibody raised against ONC-Xmrk (9).

**Construction of Expression Plasmids**—The INV-ONC chimera was generated by replacing an EcoRI fragment from pPR5 ONC (pPR5 Xmrk; Ref. 13) containing the extracellular, transmembrane, and juxtamembrane domains from the ONC-Xmrk receptor with an INV-Xmrk fragment corresponding to the same domains (6). An EcoRI-Eco47III and an Eco47III-SalI fragment from ONC-Xmrk were used to replace the corresponding fragments in pPR5 INV and thus generate the Xmrk(G359R,F580T) and Xmrk(F470L,S476N,M595I) chimeras, respectively. The different point mutations were created using a Mutagen-Phagemid in vitro mutagenesis kit (version 2, Bio-Rad). Different fragments of INV- or ONC-Xmrk cloned in pBluescript were used as templates. The following primers were used to generate the mutations: 5′-GAGAGATCATTGTTGTTGTTG3′ for the Xmrk(F580T) mutant, 5′-GAGCTGATTTGCTAGTTGTTG3′ for the Xmrk(G359R) mutant, and 5′-TGGAGCTAGTCATGTTGTTG3′ for the Xmrk(F470L) mutant. The INV-Xmrk mutant was generated by replacing a CpoI-NsiI fragment from INV-Xmrk by the corresponding one from ONC-Xmrk containing the C578S mutation. All constructs containing point mutations were sequenced to ensure that the desired mutation had been introduced.

**Cosmid Clones**—Three clones from a X. maculatus cosmid library were used for sequencing the mutations. Cosmid L11 091 contains the X-ONC-Xmrk allele, M08 036 contains the Y-ONC-Xmrk allele, and G01 008 contains INV-Xmrk (14). Oligonucleotides designed by guest on July 25, 2018http://www.jbc.org/Downloaded from

**RESULTS**

**The Mutations Responsible for Xmrk Activation Are Exclusively Located in the Extracellular Domain**—From an earlier study (6), it was known that the activation of ONC-Xmrk is due not only to overexpression but also to one or several mutations located in the extracellular domain of ONC-Xmrk that might contribute to the oncogenic potential of the receptor. It was shown that an ONC-INV chimera containing the extracellular part of ONC-Xmrk fused to the intracellular domains of INV-Xmrk is strongly autophosphorylated when transiently expressed in human 293 cells. However, there are five mutations in the carboxyl terminus of ONC-Xmrk whose role was still unknown. Although the carboxyl terminus is the most divergent region in this family of receptors, two of the substitutions found there correspond to highly conserved residues in all subclass I RTKs. One is the exchange of a proline for leucine (P984L), and the other is a tyrosine for asparagine (Y1038N) (6). To exclude a possible contribution of the intracellular domain of ONC-Xmrk in its oncogenic activation, an INV-ONC chimera containing the extracellular sequences of INV-Xmrk and the intracellular region of ONC-Xmrk was generated. After transfection of 293 cells and immunoprecipitation of the chimeric receptor, the INV-ONC chimera showed a level of phosphorylation clearly lower than the ONC-INV construct and similar to that of INV-Xmrk (Fig. 1). This indicates that all activating mutation(s) should be located in the extracellular part and none of the intracellular mutations of ONC-Xmrk is involved in activation. This was confirmed by introducing the five COOH-terminal amino acid changes (P984L, N1025T, A1035T, Y1038N, and L1156Q) into INV-Xmrk. All mutant receptors did not show enhanced autophosphorylation when compared with INV-Xmrk (data not shown).

**Evolution of Species-specific Changes**—For technical reasons, the cDNAs from INV-Xmrk and ONC-Xmrk genes were originally isolated from two different species of Xiphophorus. The cDNA from INV-Xmrk was isolated from the Xiphophorus...
indicated constructs (ONC, ONC-Xmrk; INVceptor variants. Whole cell lysates from 293 cells transfected with the receptor expression level by Western blot analysis using anti-mrk (data not shown). Receptor dimerization was subsequently analyzed with anti-phosphotyrosine (anti-ptyr) on a Western blot. Before immunoprecipitation lysates were checked for the same domain of INV and the intracellular domain of ONC) were used for immunoprecipitation (Ip) with anti-mrk. The immunoprecipitates were subsequently analyzed with anti-phosphotyrosine (anti-ptyr) on a Western blot. Before immunoprecipitation lysates were checked for the same receptor expression level by Western blot analysis using anti-mrk (data not shown).

molecular weight phosphorylated form of the molecule that, however, could not be resolved in the routine SDS-polyacrylamide gels. This form appeared exclusively in the constructs where the extracellular domain of ONC-Xmrk was present (see Fig. 1). To test whether this could correspond to a dimeric form of the ONC-Xmrk receptor, electrophoreses in gradient denaturing gels under reducing and nonreducing conditions were performed. After blotting and detection of the proteins with an Xmrk antibody for both INV- and ONC-Xmrk, the appearance of a 160-kDa form that corresponds to the Xmrk monomer was recorded (Fig. 3A, lanes 1 and 2). In addition, for ONC-Xmrk under nonreducing conditions, another signal with a higher molecular weight was appearing that was consistent with the size of a dimer. This signal was not present under reducing conditions. When the ONC-INV and INV-ONC chimeras were subjected to the same analysis, the signal corresponding to the dimer was only present in the case of the ONC-INV chimera under nonreducing conditions (Fig. 3A, lanes 3 and 4). These data point to the presence of one or several mutations in the extracellular part of ONC-Xmrk allowing ligand-independent covalent dimerization.

To investigate whether disulfide-linked dimers were also present in ONC-Xmrk from fish melanoma cells, we analyzed PSM cells and melanoma tissue extracts on a gradient gel. The

![Fig. 1. Tyrosine phosphorylation state of different Xmrk receptor variants.](http://www.jbc.org/)

**Fig. 1.** Tyrosine phosphorylation state of different Xmrk receptor variants. Whole cell lysates from 293 cells transfected with the indicated constructs (ONC, ONC-Xmrk; INV, INV-Xmrk; ONC-INV, chimera bearing the extracellular domain of ONC and the intracellular domain of INV; INV-ONC, chimera bearing the extracellular domain of INV and the intracellular domain of ONC) were used for immunoprecipitation (Ip) with anti-mrk. The immunoprecipitates were subsequently analyzed with anti-phosphotyrosine (anti-ptyr) on a Western blot. Before immunoprecipitation lysates were checked for the same receptor expression level by Western blot analysis using anti-mrk (data not shown).

**Fig. 2.** Amino acid differences between INV- and ONC-Xmrk of different origins: comparison with other members of the EGFR family. The number on the top of each residue column indicates its position in INV-Xmrk. ONC-Xmrk sequences are from PSM cells and X. maculatus. INV-Xmrk sequences are from X. maculatus or A2 cell line derived from X. xiphidium. Xegfr corresponds to Xiphophorus EGFR (see Footnote 2). Her (49), Her2 (50), Her3 (51), and Her4 (52) are the human members of the EGFR family. Mouse (53), chicken (54), Dro sophila (55), and C. elegans (56) correspond to the residues of the EGFR in these organisms.

**Fig. 3.** The extracellular domain of ONC-Xmrk promotes covalent dimerization. A, cell extracts from 293 cells expressing INV-Xmrk, ONC-Xmrk, and the ONC-INV and INV-ONC chimeras were separated on 3–8% gradient SDS-polyacrylamide gels under reducing (left panel) or nonreducing (right panel) conditions. Proteins were analyzed with an anti-mrk serum on a Western blot. Monomers and dimers of the Xmrk receptor are indicated. B, protein lysates from PSM cells and malignant Xiphophorus melanoma (mel) were electrophoresed on a 3–8% gradient SDS-PAGE gel, blotted to nitrocellulose, and detected with anti-mrk. Dimeric and monomeric forms of Xmrk are indicated.

*xiphidium*-derived A2 cell line and ONC-Xmrk gene from the PSM cell line derived from *Xiphophorus maculatus* melanoma (18). The different species origin of these two genes could account for some of the effective nucleotide differences between them. To distinguish which of the changes in the extracellular domains were due to a species-specific variation and which were potentially functional mutations of the oncogenic Xmrk, we sequenced a series of cosmid clones that contain genomic DNA from different alleles of X. maculatus INV- and ONC-Xmrk. The alignment on Fig. 2 shows that none of the nucleotide polymorphisms results in an amino acid difference between X. maculatus and X. xiphidium INV-Xmrk. However, two of the amino acid changes noted earlier (P195H and S446R) probably are irrelevant as they do not appear in the ONC-Xmrk sequence obtained from X. maculatus DNA. They may be a cell line-specific characteristic as they appear exclusively in the PSM cells. They were not considered further; thus, the number of supposed effective mutations in the extracellular region of ONC-Xmrk is reduced to six. Four of these six mutations (G359R, P470L, S476N, and M595I) involve amino acids that are not conserved in other members of the EGFR family, the fifth includes the loss of a semiconserved proline (P388T), and the sixth eliminates a highly conserved cysteine (C578S) from the second cysteine-rich domain of the receptor.

**Mutations in the Extracellular Domain Promote Covalent Dimerization—**One mechanism described for RTKs resulting in constitutive activation is ligand-independent dimerization. Using a heterologous system (293 cells) for transient expression of the different Xmrk constructs, we had observed traces of a high molecular weight phosphorylated form of the molecule that, however, could not be resolved in the routine SDS-polyacrylamide gels. This form appeared exclusively in the constructs where the extracellular domain of ONC-Xmrk was present (see Fig. 1). To test whether this could correspond to a dimeric form of the ONC-Xmrk receptor, electrophoresis in gradient denaturing gels under reducing and nonreducing conditions were performed. After blotting and detection of the proteins with an Xmrk antibody for both INV- and ONC-Xmrk, the appearance of a 160-kDa form that corresponds to the Xmrk monomer was recorded (Fig. 3A, lanes 1 and 2). In addition, for ONC-Xmrk under nonreducing conditions, another signal with a higher molecular weight was appearing that was consistent with the size of a dimer. This signal was not present under reducing conditions. When the ONC-INV and INV-ONC chimeras were subjected to the same analysis, the signal corresponding to the dimer was only present in the case of the ONC-INV chimera under nonreducing conditions (Fig. 3A, lanes 3 and 4). These data point to the presence of one or several mutations in the extracellular part of ONC-Xmrk allowing ligand-independent covalent dimerization.

To investigate whether disulfide-linked dimers were also present in ONC-Xmrk from fish melanoma cells, we analyzed PSM cells and melanoma tissue extracts on a gradient gel. The
detection with an anti-mrk serum showed, in both cases, the presence of dimers under nonreducing conditions, whereas these were not present under reducing conditions (Fig. 3B). This suggests that in vivo the same mechanism is present as studied after overexpression of ONC-Xmrk in 293 cells.

More than One Activating Mutation Is Present in the Extracellular Domain—To identify the extracellular mutation(s) present in ONC-Xmrk responsible for dimerization, additional chimeric constructs were made. In these constructs portions of the ONC-Xmrk extracellular domain were used to replace the corresponding regions in INV-Xmrk, thus introducing groups of mutations in the backbone of the proto-oncogene. Two new chimeras were constructed, Xmrk(G359R,P388T), which contains the two most amino-terminal mutations in the extracellular domain, and Xmrk(P470L,S476N,C578S,M595I), which contains the four last extracellular mutations of ONC-Xmrk. When these chimeras were subjected to Western blot analysis, dimer formation under nonreducing conditions was observed in both cases (Fig. 4), suggesting that more than one activating mutation exists in ONC-Xmrk that leads to ligand-independent receptor dimerization.

The C578S Mutation Is Involved in Dimer Formation and Constitutive Activation of Xmrk—It has been already described for RTKs (19, 20) that when one conserved cysteine involved in intramolecular disulfide bonding is lost the remaining cysteine of the pair is able to form an intermolecular disulphide bridge. This aberrant bonding leads to the formation of receptor dimers that can be observed under nonreducing conditions. In the case of ONC-Xmrk, a cysteine in position 578 is lost and substituted by a serine. To verify whether this change was one of the mutations involved in activation, an INV-Xmrk receptor containing the Cys-to-Ser mutation was constructed. After transient expression in 293 cells, the INV(C578S) mutant was immunoprecipitated with an anti-mrk serum and detected with an anti-phosphotyrosine antibody. This analysis showed increased tyrosine phosphorylation of the mutant receptor compared with INV-Xmrk, demonstrating that the introduction of this mutation was sufficient to activate Xmrk receptor (Fig. 5A). When the INV(C578S) receptor was analyzed on a denaturing gradient gel under nonreducing conditions, the appearance of a band was observed consistent with the size of the dimer as it appeared also for ONC-Xmrk (Fig. 5B). However, the extent of dimerization was lower than that observed for ONC-Xmrk, suggesting additional differences between the two receptors.

FIG. 4. Different mutations promote independently covalent dimerization of the Xmrk receptor. 293 cells transiently expressing two different constructs were lysed, and cellular proteins were resolved in 3–8% gradient SDS-polyacrylamide gel under reducing (left panel) and nonreducing (right panel) conditions. Proteins were transferred to nitrocellulose and subjected to Western blotting using an anti-mrk serum. Monomeric and dimeric forms are indicated. Lanes 1 and 3, Xmrk(G359R,P388T); lanes 2 and 4, Xmrk(P470L,S476N,C578S,M595I).

FIG. 5. Analysis of Xmrk receptor containing the C578S mutation. A, cell extracts from transiently transfected 293 cells were immunoprecipitated with anti-mrk serum and subjected to immunoblot analysis with anti-phosphotyrosine (anti-ptyr) (upper panel), and the blot was reprobed with an anti-mrk (lower panel). B and C, protein lysates of 293 cells expressing different constructs were separated on a 3–8% gradient denaturing gel under reducing (left panels) and nonreducing (right panels) conditions and analyzed on a Western blot using anti-mrk. Xmrk monomers and dimers are indicated. C, lanes 1 and 3, Xmrk(P470L,S476N,C578S,M595I); lanes 2 and 4, Xmrk(P470L,S476N,M595I).

To further limit the mutations being involved in dimer formation, a new construct was made wherein the Xmrk(P470L,S476N,C578S,M595I) chimera S578 was reverted to the wild type cysteine. The expression of the Xmrk(P470L,S476N,M595I) variant in 293 cells and subsequent analysis of the receptor protein showed that this receptor is not able to produce dimers (Fig. 5C). This finding confirms that P470L, S476N, and M595I do not contribute to ONC-Xmrk activation.
An Additional Mutation in the Extracellular Part of Xmrk Is Involved in Dimer Formation and Activation—As shown by the Xmrk(G359R,P388T) chimera, there exists a second activating mutation in ONC-Xmrk that also leads to aberrant disulfide bridging and dimerization. To find out which of these two mutations was responsible for dimer formation, the Arg mutation in position 359 was reverted to the wild type Gly. The analysis in gradient denaturing gels of 293 extracts expressing the Xmrk(P388T) mutant showed an almost complete disappearance of the dimer band, suggesting that the Arg in position 359 was the second activating mutation (Fig. 6A). To further support this, we introduced the Arg mutation in the backbone of INV-Xmrk and studied its level of phosphorylation. Cellular extracts from 293 cells transiently transfected with INV(G359R) mutant were immunoprecipitated with an anti-mrk serum and subsequently detected with an anti-mrk antibody. The INV(G359R) mutant appeared strongly phosphorylated (Fig. 6B), showing as in the case of the C578S mutation that covalent dimer formation correlates with increased receptor phosphorylation.

**Different Glycosylation Patterns due to ONC-Xmrk Mutations—**Analysis of INV- and ONC-Xmrk with the same antibody on Western blots revealed that both receptors had slightly different electrophoretic mobilities in SDS-denaturing gels. ONC-Xmrk always migrated as a smaller molecular weight form than INV-Xmrk (Fig. 7A). This different extent of migration was obviously associated with the activating mutations. The ONC-INV chimera showed the same mobility as ONC-Xmrk (data not shown). However, when the chimeras Xmrk(P470L,S476N,C578S,M595I) and Xmrk(G359R,P388T) containing only one of the activating mutations each were analyzed in Western blots, a mixture of the two forms characteristic of ONC- and INV-Xmrk appeared (Fig. 7B). When both activating mutations were reverted to the proto-oncogenic residues, the resulting Xmrk(P388T) and Xmrk(P470L,S476N,M595I) forms migrated in the SDS-denaturing gels like the proto-oncogenic form of the receptor. As all these proteins contain the same number of amino acids, this could reflect a different post-translational modification. The main post-translational modification in the extracellular domain of a receptor tyrosine kinase from the family of the EGFR is the glycosylation of certain asparagine residues. However, none of the mutations affects a canonical glycosylation sequence. It has been reported that proteins that expose reactive thiol groups can be retained in the ER (21). Because of incorrect folding, they are kept there by some kind of quality control mechanism and they are not processed further to the Golgi (22, 23). Glycoproteins stopped in this cellular compartment contain high mannose type oligosaccharides and are therefore sensitive to endoglycosidase H (endo H) digestion. In contrast, fully N-glycosylated receptors anchored in the membrane are endo H-resistant. In the case of ONC-Xmrk, we have demonstrated that the activating mutations lead to the formation of receptor dimers by intermolecular disulfide bridging between unpaired cysteines. To test whether these proteins containing activating mutations and thereby leaving active thiol groups were endo H-sensitive, we performed digestions on extracts corresponding to transient transfections of INV- and ONC-Xmrk in 293 cells. The ONC-Xmrk form of approximately 155 kDa was completely digested, and a band of about 135 kDa appeared (Fig. 7A).
mutations are present. Assessing of the Xmrk receptor is affected when the extracellular domain of Neu was inhibitory for receptor activation could not be excluded. It has been reported for the Ret receptor that the MEN2B mutation located in the catalytic domain when cloned in a EGFR/ret construct was less effective in activating the Ret function as compared with full length Ret receptor (24). The fact that the level of autophosphorylation of the INV-ONC chimera was comparable to that of INV-Xmrk when expressed in 293 cells showed that none of these mutations is responsible for the high phosphorylation level observed in ONC-Xmrk and proved the assumption that the activating mutations are exclusively located in the extracellular domain. Furthermore, none of the mutations is located in any of the described substrate binding sites of Xmrk (25). This excludes the possibility of interaction of the oncogene receptor with intracellular substrates different to those of the physiological receptor, a phenomenon already described for other RTKs (26–29).

The extracellular domain of the EGFR family members can be subdivided into four regions (1). Subdomains I and III are involved in ligand binding in the EGFR (30), subdomain III being the one containing the major ligand binding site (31). Subdomains II and IV are cysteine-rich domains characterized by the existence of highly conserved cysteines involved in intramolecular disulfide bonding (32). In the extracellular domain of ONC-Xmrk, six effective substitutions were found. These six mutations are not scattered throughout the whole extracellular region but are clustered in subdomains III and IV. Activating mutations in the extracellular domain of different RTKs have been described. Many stabilize a dimeric conformation and lead to ligand-independent stimulation of the tyrosine kinase activity. Others abolish ligand binding, and a failure in receptor down-regulation is the major mechanism that enhances tumorigenicity (33). The permanently active chimera ONC-INV showed not only a high level of autophosphorylation but was also able to form covalent dimers in the absence of a ligand due to the presence of two independent activating mutations. One of the mutations (C578S) substitutes a serine for a cysteine. By introducing this change in the backbone of INV-Xmrk, we could demonstrate that it was enough to activate the receptor through the formation of disulfide-linked dimers. Such a mechanism for receptor activation is consistent with reports on mutant Ret receptors (19, 34–36), different members of the fibroblast growth factor receptor family (reviewed in Ref. 37), Ron receptor (38), and Epo receptor (39), which all show ligand-independent dimerization and activation by mutations that create an unpaired cysteine. In most of these cases, as in ONC-Xmrk, the mutation destroys an intramolecular disulfide bond allowing cysteine and the remaining cysteine of the pair is then free to form intermolecular links. Taking the disulfide bond structure of the human EGFR as reference (32), we can predict that Cys-586 in ONC-Xmrk is the intramolecular part of the cysteine. In most of these cases, as in ONC-Xmrk, the mutation destroys an intramolecular disulfide bond allowing cysteine and the remaining cysteine of the pair is then free to form intermolecular links. Taking the disulfide bond structure of the human EGFR as reference (32), we can predict that Cys-586 in ONC-Xmrk is the intramolecular partner of the mutated cysteine and thus the unpaired residue responsible for the formation of the aberrant link.

The effect of this kind of mutation has also been described in other members of the EGFR family. Deletions affecting different cysteines within the extracellular domain of Neu were found in mammary tumors of transgenic mice carrying a mouse mammary tumor virus (MMTV)/wild type neu fusion (40). In Let-23, the Caenorhabditis elegans EGFR homolog, the sa62 mutation found in mutagenized animals involves the loss of a cysteine and leads to excess vulval differentiation (41). In the human EGFR, introduction of an extra cysteine near the transmembrane domain was enough to promote dimer formation in transient expression assays (42). The case reported here of ONC-Xmrk activation and ligand-independent dimerization by loss of a cysteine residue is the first naturally occurring and disease-related mutation of a cysteine described in the EGFR family (43).
Dimerization and Activation of Oncogenic Xmrk

It has been shown for several RTKs that receptor dimerization does not always lead to activation (35, 44, 45). It can happen that, although dimers are formed, the two receptor molecules are brought together in an incorrect spatial configuration preventing cross phosphorylation of the receptors. In the case of ONC-Xmrk, the one link that the C578S mutation provides for dimer formation is enough to activate the receptor, leading to the suggestion that this mutation can bring the receptors together in a tight and correct way. Moreover, if the physiological ligand-mediated dimer interface is maintained, additional residues that interact in natural conditions may also play a role to stabilize these aberrant dimers.

The second activating mutation found in ONC-Xmrk is the substitution of a glycine by an arginine in position 359. This mutation is located at the beginning of subdomain III of the receptor, which in the EGFR contains the major ligand binding site (31). The mechanism of action of this mutation seems to be similar to the one found for the C578S substitution, namely the formation of disulfide-linked dimers. The ability to dimerize under nonreducing conditions implicates a role for a cysteine responsible for building intermolecular bonds. This normally happens when a mutation leads to the appearance of an unpaired cysteine, but in the case of the G359R mutation, no cysteine is being lost or gained. Therefore, the more feasible explanation for the appearance of the dimers would be that this mutation provokes a structural change that could disrupt a disulfide bond enabling the cysteines involved to establish intermolecular interactions. A similar mechanism has been demonstrated for two activating mutations of FGFR2. In this case two different mutations not involving cysteines but being structurally close to a disulfide bond alter the local conformation and prevent the formation of the intramolecular bond (46). Additionally, in the case of the Ret receptor, a deletion involving Glu-632–Leu-633 promotes disulfide-linked dimer formation. This deletion is placed just upstream of Cys-634, whose substitution is responsible for the MEN2A syndrome (36).

In addition to inducing activation of the receptor, the point mutations at positions 359 and 578 were found to bring about partial inhibition of glycosylation and processing of ONC-Xmrk. The presence of only one of the activating mutations was sufficient for the appearance of an endo H-sensitive form of the molecule. In the case of ONC-Xmrk and ONC-INV, containing both activating mutations, the rate of maturation appears to be very slow due to the strong retention observed in the ER, and thus only the endo H-sensitive form is present. This leads to the conclusion that ONC-Xmrk is able to form homodimers in the ER; furthermore, it can signal from there. This is in agreement with data reported for other mutated receptors. Two different Ret receptor mutants causing MEN2A syndrome were shown to be retained in the ER and were able to signal from there (36). A similar situation was described for a splicing variant of the RON receptor (38) and for a mutant of the cytokine receptor EPO-R (39).

In conclusion, we have found that ONC-Xmrk contains two mutations that are able to activate the receptor independently. Although different activating mutations have been described in the extracellular domain of several RTKs, thus far only one activating mutation was present in each reported case. A MEN2A case caused by two de novo mutations of the RET gene was published recently, but the role of one of the mutations in activation is still not clear (47). Thus, the case of ONC-Xmrk is the first RTK described where two independently activating mutations are present and inherited.

The presence of two independent activating mutations in ONC-Xmrk is also intriguing from an evolutionary point of view. The wild-type nonhybrid fish found in nature are poly-
Borrello, M. G., and Pierotti, M. A. (1999) Oncogene 18, 4833–4838
37. Webster, M. K., and Donoghue, D. J. (1997) Trends Genet. 13, 178–182
38. Collesi, C., Santoro, M. M., Gaudino, G., and Comoglio, P. M. (1996) Mol. Cell
Biol. 16, 5518–5526
39. Watowich, S. S., Yoshimura, A., Longmore, G. D., Hilton, D. J., Yoshimura, Y.,
and Lodish, H. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2140–2144
40. Siegel, P. M., and Muller, W. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93,
8878–8883
41. Katz, W. S., Lesa, G. M., Yannoukakos, D., Clandinin, T. R., Schlessinger, J.,
and Sternberg, P. W. (1996) Mol. Cell. Biol. 16, 529–537
42. Sorskin, A., Lemmon, M. A., Ullrich, A., and Schlessinger, J. (1994) J. Biol.
Chem. 269, 9752–9759
43. Robertson, S. C., Tynan, J. A., and Donoghue, D. J. (2000) Trends Genet. 16,
265–271
44. Cao, H., Bangalore, L., Dompé, C., Bormann, B.-J., and Stern, D. F. (1992)
J. Biol. Chem. 267, 20489–20492
45. Burke, C. L., and Stern, D. F. (1998) Mol. Cell. Biol. 18, 5371–7379
46. Robertson, S. C., Meyer, A. N., Hart, K. C., Galvin, B. D., Webster, M. K., and
Donoghue, D. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4567–4572
47. Tessitore, A., Sinisi, A. A., Pasquali, D., Cardone, M., Vitale, D., Bellastella, A.,
and Colantuoni, V. (1999) J. Clin. Endocrinol. Metab. 84, 3522–3527
48. Weis, S., and Schartl, M. (1998) Genetics 149, 1909–1920
49. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A., Lee, J.,
Yarden, Y., Liberman, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, D. M., and Seeburg, P. H. (1984) Nature 309, 418–425
50. Coussens, L., Yang-Feng, T. L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J.,
Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. (1985) Science 229, 1132–1139
51. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald,
V. L., Todaro, G. J., and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87,
4965–4969
52. Plowman, G. D., Culouscu, J. M., Whitney, G. S., Green, J. M., Carlton, G. W.,
Foy, L., Neubauer, M. G., and Shoyab, M. (1993) Proc. Natl. Acad. Sci.
U. S. A. 90, 1746–1750
53. Paria, B. C., Dai, S. K., Andrews, G. K., and Dey, S. K. (1993) Proc. Natl. Acad.
Sci. U. S. A. 90, 55–59
54. Lax, I., Johnson, A., Howk, R., Sap, J., Bellot, P., Winkler, M., Ullrich, A.,
Vennstrom, B., Schlessinger, J., and Givol, D. (1988) Mol. Cell. Biol. 8,
1970–1978
55. Livneh, E., Glazer, L., Segal, D., Schlessinger, J., and Shilo, B.-Z. (1985) Cell
40, 589–607
56. Arslan, R. V., Koga, M., Mendel, J. E., Oshima, Y., and Sternberg, P. W. (1990)
Nature 348, 693–699
Ligand-independent Dimerization and Activation of the Oncogenic Xmrk Receptor by Two Mutations in the Extracellular Domain
Ana Gómez, Claudia Wellbrock, Heidrun Gutbrod, Nicola Dimitrijevic and Manfred Schartl

J. Biol. Chem. 2001, 276:3333-3340.
doi: 10.1074/jbc.M006574200 originally published online October 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006574200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 28 of which can be accessed free at http://www.jbc.org/content/276/5/3333.full.html#ref-list-1