MADR2 Maps to 18q21 and Encodes a TGFβ–Regulated MAD–Related Protein That Is Functionally Mutated in Colorectal Carcinoma

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Summary

The MAD–related (MADR) family of proteins are essential components in the signaling pathways of serine/threonine kinase receptors for the transforming growth factor β (TGFβ) superfamily. We demonstrate that MADR2 is specifically regulated by TGFβ and not bone morphogenetic proteins. The gene for MADR2 was found to reside on chromosome 18q21, near DPC4, another MADR protein implicated in pancreatic cancer. Mutational analysis of MADR2 in sporadic tumors identified four missense mutations in colorectal carcinomas, two of which display a loss of heterozygosity. Biochemical and functional analysis of three of these demonstrates that the mutations are inactivating. These findings suggest that MADR2 is a tumor suppressor and that mutations acquired in colorectal carcinomas may function to disrupt TGFβ signaling.

Introduction

An important step in the development of malignant tumors may involve the loss of sensitivity to negative growth regulators. TGFβ, a potent natural antiproliferative agent, is believed to play an important role in suppressing tumorigenicity. Comparisons of human colon carcinoma and melanoma cell lines have demonstrated a progressive loss of responsiveness to TGFβ growth-inhibitory effects as tumor aggressiveness increases (reviewed by Filmus and Kerbel, 1993; Roberts and Sporn, 1993). Thus, an understanding of the molecular events associated with loss of TGFβ responsiveness in tumors could provide major insights into the general mechanisms underlying the development of malignancies. At present, the mechanism for the escape from TGFβ regulation is not clear; however, mutational inactivation of components of TGFβ signaling pathways could be one mechanism underlying acquisition of TGFβ resistance.

TGFβ signals through heteromeric receptor complexes of type I (TβR I) and type II (TβR II) serine/threonine kinase receptors (reviewed by Massagué et al., 1994; Miyazono et al., 1994). Receptor activation occurs upon binding of ligand to TβR II, which then recruits and phosphorylates TβR I, which propagates the signal to downstream targets (Chen and Weinberg, 1995; Wrana et al., 1994). A pivotal role for the type I receptor in propgating signals is supported by the observation that constitutively active type I receptors are capable of signaling biological responses in the absence of both ligand and type II receptors (Attisano et al., 1996; Hoodless et al., 1996; Nellen et al., 1996; Wieser et al., 1995). Consistent with this, the specificity of cellular responses to TGFβ superfamily members also appears to be mediated by the type I receptors. Thus, TGFβ and activin signal similar antiproliferative and gene responses through their highly related type I receptors (Cárcamo et al., 1994).

Several studies have indicated that alterations in TGFβ receptor expression or function may be involved in some cancers. For example, in a subset of colon cancer cell lines that display high rates of microsatellite instability and in several TGFβ–resistant human gastric cancers, genetic changes in the type II receptor have been identified (Markowitz et al., 1995; Park et al., 1994). However, since intracellular targets of the TGFβ receptors are poorly understood, the importance of disrupting TGFβ signaling pathways in promoting tumorigenesis is unknown.

Recently, MADs (mothers against dpp) and MADR (MAD–related) proteins have been identified in a variety of species as important components of the signal transduction pathway that are required for serine/threonine kinase receptor signaling (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Newfeld et al., 1996; Savage et al., 1996; Sekelsky et al., 1995; Thomsen, 1996; Wiersdorff et al., 1996). Drosophila MAD and the highly related vertebrate MADR1 appear to be essential for signaling of DPP/BMP2 pathways and can specify bone morphogenetic protein (BMP)–specific biological responses (Graff et al., 1996; Hoodless et al., 1996; Newfeld et al., 1996; Thomsen, 1996; Wiersdorff et al., 1996). MAD proteins, although highly conserved across species, do not contain any known structural motifs; thus, it is difficult to predict their precise mode of action (reviewed by Massagué, 1996; Wrana and Attisano, 1996). However,

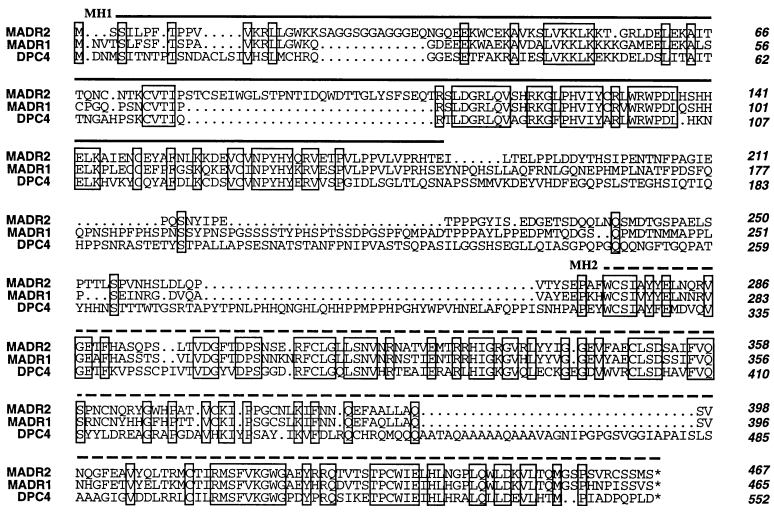

Figure 1. Sequence Comparison of Mammalian MAD–Related Proteins

Alignment of the predicted amino acid sequences of human MADR1, MADR2, and DPC4. Residues conserved in all three sequences are boxed, and the MH1 and MH2 regions at the amino- and carboxyl-termini are indicated (MH1, solid overline; MH2, broken overline). Gaps introduced to maximize alignment are shown as dots, and the amino acid residues are numbered on the right.

MADR1 is rapidly and specifically phosphorylated by BMP2 and not TGFβ–induced pathways (Hoodless et al., 1996). Furthermore, MADR1 redistributes from the cytoplasm to the nucleus upon induction of signaling, suggesting that MADs may have a nuclear function (Hoodless et al., 1996; Liu et al., 1996) and may act as transcriptional activators (Liu et al., 1996). Recently, a search for tumor suppressor genes implicated in pancreatic cancer led to the identification of the MAD–related gene, DPC4 (Hahn et al., 1996a). However, the signaling pathway in which DPC4 functions is unknown.

In this study, we demonstrate that MADR2 is rapidly phosphorylated by activation of TGFβ signaling pathways. The gene for MADR2 maps close to DPC4 at 18q21, a region often deleted in human cancers (Vogelstein et al., 1988; Yamaguchi et al., 1992). Analysis of MADR2 in sporadic colorectal carcinoma has identified four missense mutations in MADR2, two of which are associated with a loss of heterozygosity (LOH). Biochemical analysis indicates that three of these missense mutations lead to either a loss of protein expression or loss of TGFβ–regulated phosphorylation. Furthermore, functional analysis of mesoderm induction in Xenopus embryos demonstrates that these three mutations also lead to inactive protein. Thus, MADR2, a component of the TGFβ signaling pathway, is functionally mutated in tumors and is implicated as a tumor suppressor gene in colorectal carcinoma. Together with the suggested involvement of DPC4 in pancreatic cancer, these findings indicate that MADs may represent a new class of tumor suppressor genes important in human cancer.

Results

Identification of a MAD–Related Protein, MADR2

MAD and MAD–related proteins identified in Drosophila (MAD), Caenorhabditis elegans (sma-2, sma-3, sma-4), Xenopus (Xmad1 and Xmad2), and humans (MADR1, DPC4) have been demonstrated to play a crucial role in signal transduction by members of the TGFβ superfamily (reviewed by Massagué, 1996; Wrana and Attisano, 1996). To clone a MAD–related molecule that might function specifically in the TGFβ pathway, we searched a database of expressed sequence tags (Lennon et al., 1996). A pair of nonoverlapping partial clones were identified that contained open reading frames that displayed similarity either to the amino- or the carboxyl-terminus of MADR1. To obtain the full-length coding sequence, we designed primers encoding the predicted start and stop codons for polymerase chain reaction (PCR) and used a human kidney cDNA library as template. A contiguous sequence of 1605 base pairs was obtained, which included an open reading frame of 467 amino acids (Figure 1). The predicted protein is related to MAD and MADR1, and, thus, we have termed this protein MADR2. An alignment of MADR2 with the other mammalian MAD–related molecules indicates that it is more closely related to MADR1 than to DPC4, a candidate tumor suppressor gene in pancreatic cancer (Hahn et al., 1996a). Further, MADR2 is highly related (98% identity) to the Xenopus homolog, XMad2 (data not shown; Graff et al., 1996). As described for MADR1, the highest degree of conservation between MADR2 and the other MADs lies in the MH1 and MH2 domains (Figure 1).

Phosphorylation of MADR2 by TGFβ Signaling Pathways

Since MADR1 functions in BMP2 signaling pathways and is regulated by phosphorylation (Hoodless et al., 1996), we sought to determine which serine/threonine kinase receptor–activated pathways might regulate MADR2. To examine this, we utilized constitutively active type I serine/threonine kinase receptors. These type I receptors, generated by the introduction of a charged residue in the highly conserved GS domain (Attisano et al., 1996; Hoodless et al., 1996; Wieser et al., 1995), signal in the absence of ligand and type II receptors (Wieser et al., 1995). To facilitate our analysis, we constructed a mammalian expression vector that encoded a Flag epitope tag at the amino-terminus of MADR2 (FlagMADR2). To explore whether MADR2 was a target of TGFβ or BMP signaling pathways, COS cells were transiently transfected with FlagMADR2 alone or together with wild-type or activated TβRI or ALK-3 (a BMP type I receptor). FlagMADR2 was isolated by immunoprecipitation from [^{32}P]phosphate-labeled transfectants.

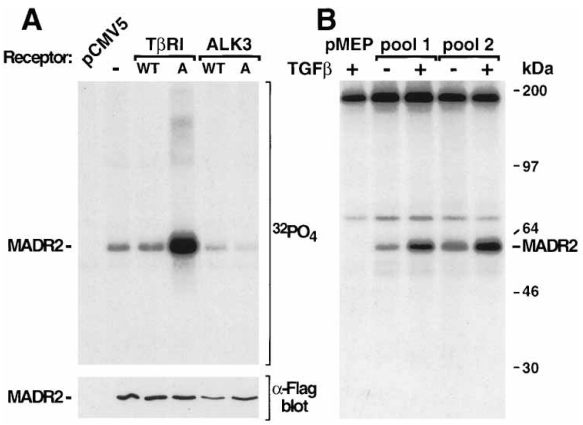

Figure 2. MADR2 Phosphorylation Is Induced by TGFβ

(A) Regulation of MADR2 phosphorylation by constitutively active type I receptors in COS-1 cells. COS-1 cells were transiently transfected with empty vector (pCMV5), FlagMADR2 alone, or FlagMADR2 with the indicated wild type (WT) or constitutively active (A) type I receptors. Transfected cells were labeled with [^{32}P]phosphate, and FlagMADR2 was purified from cell lysates by immunoprecipitation using anti-Flag antibodies and analyzed by SDS–PAGE and autoradiography (^{32}PO4). Total MADR2 protein was determined by immunoblotting total cell lysates obtained from unlabeled cultures that were prepared in parallel using anti-Flag monoclonal antibody (α-Flag blot). The migration of MADR2 is indicated on the left.

(B) TGFβ–dependent phosphorylation in Mv1Lu epithelial cells. Mv1Lu cells were stably transfected with empty vector (pMEP4) or with FlagMADR2 under the control of a metallothionein-inducible promoter and two independent pools isolated. Cells were induced overnight with 50 μM Zn^{2+}, labeled with [^{32}P]phosphate in the absence (−) or presence (+) of 100 pM TGFβ for 15 min and MADR2 phosphorylation analyzed as described in (A). The migration of MADR2 is indicated on the right.

Analysis of the immunoprecipitates revealed that phosphorylation of MADR2 was unaffected by the coexpression of wild-type TβRI, wild-type ALK-3, or activated ALK-3 (Figure 2). In contrast, MADR2 phosphorylation was substantially increased in cells cotransfected with activated TβR I. Tryptic phosphopeptides from this sample yielded one major novel phosphopeptide, indicating that phosphorylation is specific (M. Macias-Silva, L. A., and J. L. W., unpublished data). Immunoblotting with antiflag antibody of total cell lysates prepared in parallel confirmed that approximately equivalent amounts of MADR2 protein were examined (Figure 2). In a parallel analysis of MADR1, increased phosphorylation was detected only in cells cotransfected with constitutively active ALK-3, as shown previously (data not shown; Hoodless et al., 1996).

To determine whether MADR2 phosphorylation was regulated in a TGFβ–dependent manner, we stably transfected mink lung (Mv1Lu) epithelial cell lines with FlagMADR2 under the control of an inducible-metallothionein promoter (Wrana et al., 1992). Mv1Lu epithelial cells express both type I and type II receptors and are TGFβ–sensitive. Several independent pools of cells expressing FlagMADR2 in a zinc-inducible manner were generated, and the regulation of MADR2 phosphorylation in response to TGFβ was examined in two pools. Analysis of MADR2 immunoprecipitated from [^{32}P]phosphate-labeled cells that were untreated or treated with TGFβ for 15 min revealed a ligand-dependent phosphorylation in both pools (Figure 2). Thus, brief treatment of epithelial cells with TGFβ induces phosphorylation of MADR2.

Together, these data indicate that MADR2 is regulated by the TGFβ and not BMP receptor signaling pathways and, along with the known function of MAD proteins in serine/threonine kinase receptor signal transduction, suggest that MADR2 plays a role in mediating TGFβ signals.

Regional Localization of MADR2 to Chromosome 18q21 Near DPC4 and DCC

The involvement of MADR2 in the TGFβ signaling pathway suggested that this gene might function as a tumor suppressor. To investigate this possibility, we determined the chromosomal localization of *MADR2* using a combination of radiation hybrid (RH) mapping, yeast artificial chromosome (YAC) contig analysis, and fluorescence in situ hybridization. PCR primers designed from the 3′-UTR of *MADR2* were initially used to screen the GeneBridge 4 RH panel (Walter et al., 1994), and the results indicated that the gene resides on chromosome 18q21 approximately 9.3 cR from D18S460. The same primers were also used to screen the Centre d'Etudes du Polymorphisme Humain megaYAC library (Chumakov et al., 1995), and five YACs (739a3, 749d11, 887e9, 929d7, 940e10) were identified. These clones have been shown previously by the Whitehead Institute–MIT Center for Genome Research to map within a well-defined YAC contig on chromosome 18 (the contig was named WC18.5). This contig encompasses the same DNA marker, D18S460, which we linked to *MADR2* on the RH map (Hudson et al., 1995), and further analysis of our YACs identified three clones that contained both *MADR2* and D18S460 (Figure 3). The cytogenetic position of *MADR2* was confirmed further by fluorescence in situ hybridization mapping two gene-specific P1-derived artificial chromosome clones (66i18 and 201n19) to 18q21 (S. W. S., unpublished data).

The observation that *MADR2* mapped to chromosome 18q21 prompted us to determine its relative location with respect to *DPC4* and *DCC*, which also map to 18q21 (Hahn et al., 1996a) and have been shown to be frequently deleted in pancreatic cancer (Hahn et al., 1996a, 1996b) and colon cancer (Fearon et al., 1990; Vogelstein et al., 1988), respectively. Accordingly, gene-specific sequence-tagged sites (STSs) for *DPC4* and *DCC* were tested against the RH panel and all of the YAC clones from the WC18.5 contig. Using the previously published YAC and RH map (Hudson et al., 1995) as a framework, we could establish the following order: 18cen-*MADR2*-*DPC4*-*DCC*-18qter (Figure 3). Both the YAC and RH data for the genes were in complete agreement. However, the precise physical distance separating the genes was difficult to establish, since the map was based only on STS content and because two gaps are present within the YAC contig (Figure 3). Nevertheless, it was evident that MADR2 mapped to the same cytogenetic band as *DPC4* and *DCC* and within close enough physical proximity to suggest that it may also be frequently deleted in tumors demonstrating LOH of 18q21.

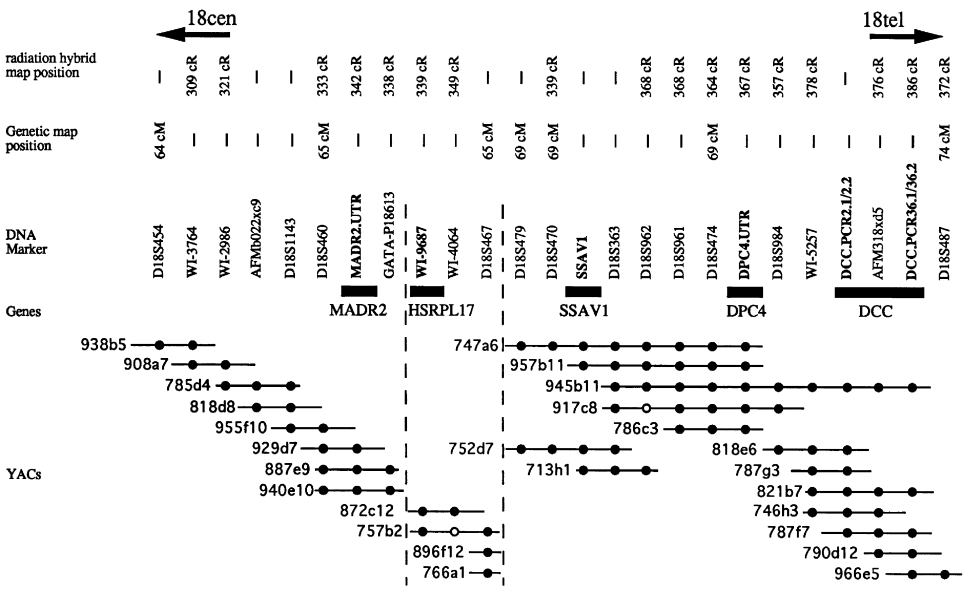

Figure 3. The Physical Position of MADR2 at Chromosome 18q21

(Top to bottom) The position of *MADR2*, *DPC4*, and *DCC* within the context of the Whitehead Institute RH framework map (Hudson et al., 1995) and the Genethon genetic linkage map is shown. *MADR2*, *DPC4*, and *DCC* are shown to map 9.32 cR, 10.31 cR, and 8.0 cR from D18S460, D18S984, and WI-5257, respectively. The RH data was used to estimate the location of the genes, but their exact position with respect to other markers was determined by YAC contig analysis. An STS contig map of a subset of YAC clones from the Whitehead Institute–MIT Center for Genome Research contig WC18.5 (Hudson et al., 1995) allowed more precise ordering of the three genes and surrounding markers. Closed circles on the YACs indicate the DNA markers we determined to be positive, while open circles represent markers that were expected to be positive but were not. The two vertical broken lines delineate gaps in the YAC contig. YACs 766a1, 785d4, 786c3, 787f7, 787g3, 887e9, 896f12, 908a7, 938b5, 940e10, and 955f10 are chimeric. DNA marker DCC. PCR2.1/2.2 and DCC. PCR36.1/36.2 represent the 5′- and 3′-end of *DCC*, respectively. The STS content data of the YACs suggests the orientation of DCC, which spans approximately 1.3 Mb of DNA (Cho et al., 1994) along the chromosome, to be 18cen-5′-DCC-3′-18qter. *MADR2* has been shown to map to 18q21 in this study, *DPC4* maps to 18q21.1 (Hahn et al., 1996a), and *DCC* maps to 18q21.3 (GenBank Database).

MADR2 Is Mutated in Colorectal Carcinoma

The localization of *MADR2* to 18q21 led us to investigate whether mutations in this gene might play a role in the development of some forms of cancer. To explore this possibility, we examined a variety of human tumors for mutations in *MADR2* using single-strand conformation polymorphism (SSCP) analysis of cDNA. We focused on the MH1 and MH2 domains of MADR2, which display the highest degree of similarity among members of the MAD family. Furthermore, it is within the MH2 domain that all of the identified mutations in *DPC4* reside (Hahn et al., 1996a). Our initial screen of 101 axillary node–negative breast carcinomas and 76 sarcomas (which included 35 osteosarcomas) did not reveal any mutations, with the exception of a single benign polymorphism at an arginine residue in one breast cancer sample (nucleotide 1245: A→G). However, in a screen of 66 sporadic colon carcinomas, we identified four tumors that had missense mutations in MADR2 (6%). In none of the samples did we detect any other neutral polymorphisms. For three of the mutations, alterations occurred in highly conserved residues within the MH2 domain and involved two nonconservative (P445H and L440R) and one conservative (D450E) change (Figure 4; Table 1). The fourth missense mutation mapped to the MH1 domain and resulted in the alteration of a highly conserved arginine residue to a cysteine (R133C; Figure 4; Table 1). In the three cases in which adjacent normal colorectal tissue was available (213, 357, and 348), MADR2 was found to have wild-type sequence, indicating that these mutations were acquired as somatic events. These latter studies also served to demonstrate that MADR2 is expressed in normal human colorectal tissue. We also examined SSCP band intensities. Comparison of mutant and wild-type alleles in each tumor sample showed that tumors 357 and 369 had little or no wild-type message, suggesting LOH at this locus. To date, we have not found evidence for germline mutations of MADR2 in a panel of lymphoblastoid lines generated from 15 patients who had a strong family history of colorectal tumors or who presented with colorectal cancer at a young age. Together, these data show that mutations in *MADR2* are specifically associated with sporadic colorectal carcinoma and suggest that *MADR2* is a candidate tumor suppressor at 18q21.

MADR2 Containing Missense Mutations Are Not Regulated by TGFβ

Mutation of a conserved residue within the MH2 domain of MADR1 has been shown to disrupt regulation by the BMP2 signaling pathway and correlates with the loss of functional protein (Hoodless et al., 1996; Savage et al., 1996; Sekelsky et al., 1995). To determine the potential consequences of the missense mutations we identified in the MH2 domain of MADR2, we investigated the regulation of the mutant proteins by the TGFβ signaling pathway. To test this, we introduced the mutations L440R,

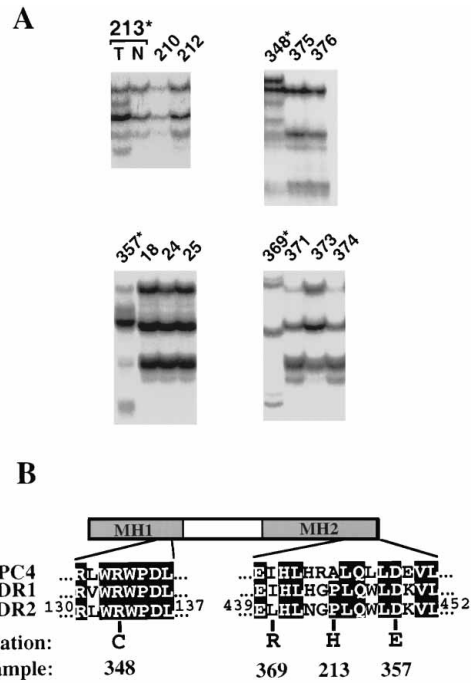

Figure 4. MADR2 Mutations Present in Colorectal Tumor Samples

(A) SSCP analysis of MADR2. Mutation 348 is from region 1, and mutations 213, 357, 369 are from region 3. Sample 213 is represented by T (tumor) and N (normal colon tissue). In each case, the mutant is followed by cases with wild-type SSCP banding patterns that were run in adjacent lanes.

(B) Summary of predicted amino acid alterations resulting from MADR2 mutations. The amino acid sequence of MADR2, from amino acid 130 to 137 of the MH1 domain and from amino acid 439 to 452 of the MH2 domain, are aligned with the corresponding regions in MADR1 and DPC-4. Conserved sequences are highlighted (solid box). The location of the missense mutations and the predicted amino acid changes are indicated.

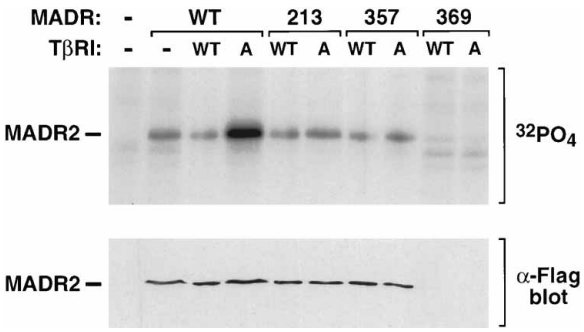

Figure 5. Analysis of TGFβ–Regulated Phosphorylation of MADR2 Harboring Missense Mutations

COS-1 cells were transiently transfected with empty vector (−), FlagMADR2 wild-type (WT) alone, or FlagMADR2 wild-type or mutated (213, 357, or 369) together with wild-type (WT) or constitutively active (A) TβRI. Transfected cells were labeled with [^{32}P]phosphate, and FlagMADR2 was purified from cell lysates by immunoprecipitation using anti-Flag antibodies and was analyzed by SDS–PAGE and autoradiography (^{32}PO4). Total MADR2 protein was determined by immunoblotting of total cell lysates using anti-Flag monoclonal antibody (α-Flag blot). The migration of MADR2 is indicated on the left. Note that the apparent change in migration on the Western blot is a gel artifact and is not reproducible.

P445H, and D450E into wild-type FlagMADR2 and expressed the protein in COS-1 cells together with either wild-type or constitutively active TβRI. Relative phosphorylation levels were assessed by immunoprecipitation of MADR2 protein from [^{32}P]phosphate-labeled cells and quantitating protein levels by Western blotting of whole-cell lysates. When wild-type MADR2 was coexpressed with activated TβRI, typical elevations in phosphorylation of MADR2 were observed (Figure 5). In contrast, two of the MADR2 mutants, 213 and 357 (P445H and D450E, respectively), showed no alterations in relative phosphorylation upon coexpression with activated receptors. Thus, both the nonconservative P445H and the conservative D450E mutations disrupt the regulation of MADR2 phosphorylation. In several attempts to express the 369 mutant (L440R) in COS cells, we were unable to detect any stable protein expression (Figure 5). This may indicate that the L440R mutation disturbs the stability of the nascent protein or interferes with translation of the mRNA. These results provide strong evidence that the missense mutations in *MADR2*, identified in colorectal carcinomas, lead to disruption of TGFβ regulation and may lead to the concomitant loss of TGFβ sensitivity in the target cells.

MADR2 Mutants Are Biologically Inactive in Xenopus Mesoderm Induction Assays

Xenopus blastula stage ectoderm can differentiate into mesodermal tissues in response to particular members of the TGFβ superfamily (reviewed by Kessler and Melton, 1994; Wall and Hogan, 1994). For example, BMPs induce ventral mesoderm such as blood, whereas activin induces dorsal types of mesoderm such as muscle and notochord. Similar to the common biological responses observed for activin and TGFβ in mammalian cells (Cárcamo et al., 1994), TGFβ can also induce dorsal mesoderm in caps ectopically expressing TGFβ type II receptor (Bhushan et al., 1994). Further, overexpression of a Xenopus homolog of TβRI that is 98% identical to TβRI in the kinase domain similarly results in formation of dorsal mesoderm (Mahony and Gurdon, 1995). Interestingly, overexpression of the Xenopus MAD–related proteins, XMad1 and XMad2, in the ectoderm mimics these effects. Thus, XMad1 (or XMad) induces ventral

Table 1. Missense Mutations in MADR2

Sample	Codon	Mutation	Amino Acid Change	Normal Tissue	Effect
213	445	CCT→CAT	Pro→His	wild-type	no phosphorylation
369	440	CTT→CGT	Leu→Arg	wild-type	unstable protein
357	450	GAC→GAG	Asp→Glu	N/A	no phosphorylation
348	133	CGC→TGC	Arg→Cys	wild-type	not done

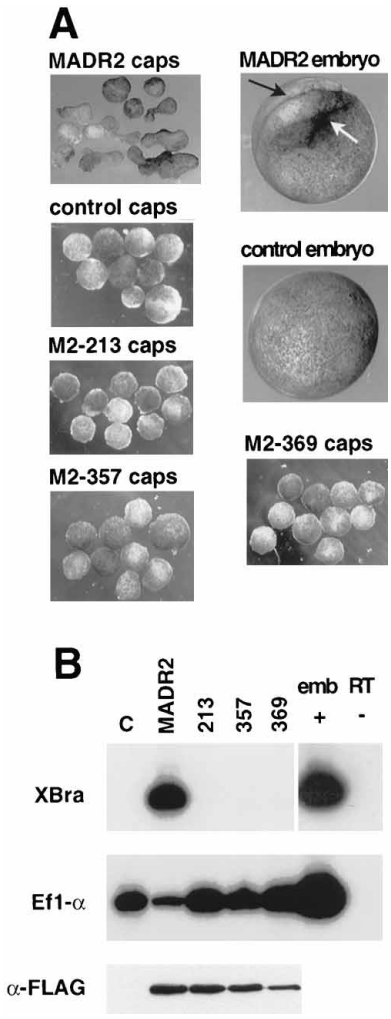

Figure 6. Induction of Morphogenetic Movements and Mesoderm by Wild-Type But Not Mutant MADR2 Proteins

(A) Animal caps cut from embryos injected with wild-type MADR2 caps elongate as they undergo morphogenetic movements associated with mesoderm differentiation, but control caps and ones expressing mutant MADR2 proteins do not. Intact embryos expressing mutant and wild-type MADR2 in the animal pole were also scored for ectopic sites of gastrulation. A representative control embryo is shown, and it is indistinguishable from embryos that expressed the mutant forms of MADR2. An embryo expressing wild-type MADR2 shows an additional invagination furrow (white arrow) whose time of appearance coincided with the formation of the dorsal lip of the Spemann organizer, the normal site of gastrulation initiation. The normal gastrulation furrow (black arrow) of the embryo pictured here is displaced from its usual more vegetal position, because of the action of the ectopic furrow. All wild-type MADR2–injected embryos displayed the phenotype shown (for all sets of embryos, n > 12). The embryos analyzed were siblings of those used in the animal cap assays in (B), all of which expressed MADR2 proteins.

(B) RT–PCR analysis on animal caps injected with wild-type and mutated MADR2 mRNAs show that only wild-type, and not mutated, MADR2 induces mesoderm as scored by the expression of brachyury, a general mesoderm marker at early to mid-gastrulation. Caps were injected with control (C; pGEM vector transcripts) and wild-type or mutant MADR2 mRNAs as indicated. EF1-α expression was scored as a general marker for RNA recovery. The emb RT plus and minus lanes are positive and negative controls, using total embryonic RNA that was (+) or was not (−) reverse-transcribed. A Western blot of animal cap proteins stained with an anti-Flag antibody is shown in the lower panel. The Western blot confirms that the various MADR2 proteins were synthesized in each set of caps, although mutant 369 displayed a lower level of expression. Animal caps (10) were pooled for each sample analyzed by Western blot, and two cap equivalents were loaded per lane. Approximately 24 caps were cut at late blastula (stage 8); at mid-gastrula (stage 12), half were harvested for RT–PCR and half for protein analysis.

mesoderm, while XMad2, like TGFβ or activin, yields dorsal types of mesoderm (Graff et al., 1996; Thomsen, 1996).

Since the protein sequence of human MADR2 is 98% identical to XMad2 (data not shown), we examined the effect of MADR2 expression in Xenopus embryos. MADR2 could induce mesoderm and trigger morphogenetic movements in a fashion similar to that of XMad2 (Figure 6). Expression of MADR2 from microinjected mRNA caused Xenopus ectoderm explants, or animal caps, to elongate (Figure 6A), as observed when TβRII-expressing caps are treated with TGFβ (Bhushan et al., 1994). These shape changes are considered to reflect the cellular movements that normally occur in the dorsal embryonic mesoderm during gastrula and neurula stages. MADR2 could also trigger ectopic gastrulation movements in the ectoderm of intact embryos (Figure 6A) precisely at the time that normal gastrulation movements began on the dorsal side of the embryo in the Spemann organizer. The MADR2 mutants 213, 357, and 369, however, did not cause animal caps to elongate or produce ectopic gastrulation movements in embryos, suggesting that the mutant proteins are functionally inactive.

We also tested wild-type and mutant MADR2 proteins for their capacity to induce mesoderm in animal caps. Wild-type MADR2 induced strong expression of the general mesodermal marker gene, brachyury (Xbra; Smith et al., 1991), but the mutants did not (Figure 6B). The lack of activity observed for the MADR2 mutants was not due to a lack of protein expression. Western blot analysis of MADR2 protein expression in injected animal caps showed that wild-type and mutant MADR2 were produced at nearly equal levels, except for 369, which was expressed at lower levels consistent with its apparent instability in mammalian tissue culture cells. These observations demonstrate that the mutations detected in colorectal tumors disrupt regulation by the TGFβ signaling pathway and yield biologically inactive MADR2 protein.

Discussion

MAD and MAD–related proteins are a novel family of proteins that function downstream of serine/threonine kinase receptors to transduce signals for members of the TGFβ superfamily (reviewed by Massagué, 1996; Wrana and Attisano, 1996). In this study, we report the characterization of MADR2 as an intracellular component of the TGFβ signaling pathway. We show that it maps to a tumor suppressor locus at 18q21 and that the gene is mutated in approximately 6% of sporadic colorectal carcinomas. We demonstrate that these mutations disrupt either the regulation of MADR2 by TGFβ

signaling pathways or the stable expression of the protein. Furthermore, using a Xenopus model system, we show that these mutations lead to inactivation of MADR2 protein function.

MADR2 Functions in the TGFβ Signaling Pathway

Genetic studies in Drosophila and C. elegans have demonstrated that MADs are required for signaling by receptors for the TGFβ superfamily (Hoodless et al., 1996; Newfeld et al., 1996; Savage et al., 1996; Wiersdorff et al., 1996). MADR1 has been shown previously to be specifically regulated by BMPs (Hoodless et al., 1996), and in this study, we show that MADR2 is regulated by TGFβ signaling pathways. These results demonstrate that at least in the case of MADR1 and MADR2, these molecules lie in specific serine/threonine kinase receptor signaling pathways. Studies in Xenopus further highlight this specificity, since overexpression of specific MADs can recapitulate the biological response normally induced by activation of the upstream receptors. Hence, XMad1 signals BMP–like responses, while XMad2/MADR2 signals TGFβ/activin–like responses (this study; Graff et al., 1996; Thomsen, 1996). Thus, MADs play a central role in determining cellular responses to TGFβ–like factors, and mutations that disrupt MADR2 function are likely to result in specific defects in TGFβ signaling.

MADR2 Is a Tumor Suppressor

Tumor suppressor genes are often inactivated when one allele acquires a somatic mutation and the second allele is lost, typically through deletion (Cavenee et al., 1983). LOH at 18q21 has been identified in numerous human cancers (Vogelstein et al., 1988; Yamaguchi et al., 1992). We mapped *MADR2* to this region of the human genome, raising the possibility that *MADR2* acts as a tumor suppressor. Our random screen of 76 sarcomas, 101 breast carcinomas, and 66 sporadic colorectal carcinomas identified four mutations specific to colon carcinoma, of which at least three were acquired somatically. In two of the samples, we could clearly detect wild-type MADR2. Since this may be due to the presence of normal tissue in the specimen or to tumor heterogeneity, this precluded a determination of potential LOH in these individuals. Nonetheless, in two of our cases, there was very low expression or loss of the normal allele suggestive of an LOH of MADR2. Together with the observation that these mutations are inactivating, these results provide strong evidence that MADR2 is a tumor suppressor gene in sporadic colorectal cancers. Recently, Riggins et al. (1996) also reported the presence of a genetic alteration in a colorectal tumor of a gene, JV18-1, which is likely to be the same as MADR2 described here.

Missense Mutations in MADR2 Are Functionally Disrupted

Our analyses of the regulation of MADR2 by TGFβ signaling pathways demonstrate that the somatic mutations we have characterized can lead to two distinct defects. Characterization of the nonconservative L440R mutation revealed that this change results in a defect in stable expression of MADR2 protein both in mammalian epithelial cells and in Xenopus embryos. While we have not determined whether this is due to a defect in translation or stability of the protein product, this mutation may result in loss of MADR2 expression in target cells. In contrast, two of the mutations in the MH2 domain characterized from tumors disrupt the regulation of MADR2 phosphorylation by TGFβ signaling pathways. Studies on MADR1 have previously identified a mutation within the MH2 domain that blocks phosphorylation by the BMP signaling pathways (Hoodless et al., 1996). This particular mutation generates null phenotypes in Drosophila or C. elegans, providing evidence that these types of mutations yield nonfunctional protein products (Savage et al., 1996; Sekelsky et al., 1995). We also observed similar inactivation of MADR2 protein function in Xenopus embryonic assays. Wild-type MADR2 induces mesoderm and morphogenetic movements similar to that seen with Xenopus Mad2, and these activities are abolished for mutant MADR2 proteins. These findings suggest that alterations within the MH2 domain of MADRs can disrupt regulation of phosphorylation and indicate that phosphorylation is important for MADR function.

Disruption of MADR2 and TGFβ Signaling in Tumorigenesis

Regardless of the mechanism, the MADR2 mutations we have identified in colorectal carcinoma yield nonfunctional protein. Thus, these mutations should lead to a similar loss of activity in the developing tumors. This might lead to a disruption of TGFβ signaling and thereby result in a loss of TGFβ sensitivity in the target cells. Since TGFβ is a potent antiproliferative factor for a broad range of epithelial cells, mutations in signaling components of this pathway may thus allow tumor cells to escape this regulation. This would clearly give neoplastic clones a growth advantage in the tissue. Thus, loss of MADR2 function may make a major contribution to the development of cancer.

18q21 Is a Locus for Multiple Tumor Suppressor Genes

LOH at 18q21 is frequently detected in many human cancers (Fearon et al., 1990; Vogelstein et al., 1988; Yamaguchi et al., 1992). Interestingly, *DPC4*, another MAD–related gene, has also been mapped to 18q21 in close proximity to MADR2 and has been identified as a candidate tumor suppressor in pancreatic carcinoma (Hahn et al., 1996a). A similar situation exists for adenomatous polyposis coli and mutated-in-colon cancer on chromosome 5q21 (Joslyn et al., 1991; Kinzler et al., 1991). Adenomatous polyposis coli mutations contribute to the development of sporadic and familial colorectal cancers; and both adenomatous polyposis coli and mutated-in-colon cancer are located in close physical proximity, share significant sequence homology with coiled-coil class proteins and are frequently deleted concurrently in many different cancers. It is interesting to speculate on the functional relationship between DPC4 and MADR2. DPC4 may function downstream of a different TGFβ superfamily member whose function is important in controlling the growth of pancreatic cells. However, it is also possible that DPC4 might also function

in the TGFβ signaling pathway, as suggested recently for the multiple sma genes in BMP signaling in C. elegans (Savage et al., 1996). If the latter is the case, it raises the possibility that MADR2 and DPC4 together may function in a cooperative way and that mutations in either gene would allow cells to escape from TGFβ sensitivity. Thus, both MADR2 and DPC4 could be implicated as tumor suppressor genes in a variety of tumors of the gastrointestinal tract.

Experimental Procedures

Construction of Expression Vectors

Several clones displaying similarity to MADR1 (clone ID numbers 136422, 145032, and 138604) were identified from the expressed sequence database (I.M.A.G.E.; Lennon et al., 1996). A pair of clones (ID numbers 136422 and 145032) were obtained and sequenced (Sequenase™ 2.0 Kit, US Biochemicals). Both encoded MADR2 from nucleotides −40 to +551. A single clone (ID 138604) appeared to encode a stop codon. To obtain a full-length cDNA for MADR2, sense and antisense primers containing convenient restriction sites for subcloning were designed based on sequence obtained from the expressed sequence database clones. To allow introduction of an epitope tag into the amino-terminus of MADR2, the start methionine in the sense primer was replaced with a SalI site and a glutamine residue to allow direct subcloning into pCMV5-Flag (Hoodless et al., 1996). PCR was performed with a human kidney cDNA library as template (Clontech, pGAD424 library). To obtain the full cDNA coding sequence, two PCR–generated constructs were completely sequenced. To generate FlagMADR2 harboring mutations, a fragment of MADR2 was amplified by PCR using cDNA prepared from colorectal carcinoma RNA samples as template. The amplified region was subcloned into the full-length FlagMADR2 in pCMV5 and the presence of the missense mutations confirmed by sequencing. For the generation of stable transfectants, FlagMADR2 was subcloned into pMEP4 (Invitrogen) behind a metallothionein-inducible promoter using convenient sites in the vector polylinker (Wrana et al., 1992).

Cell Lines and Transfections

COS-1 cells were maintained and transfected using the diethylaminoethyl-dextran as described (Hoodless et al., 1996). The Mv1Lu mink lung cells (CCL-64) expressing FlagMADR2 in pMEP4 (Invitrogen) were generated by transfection with lipofectin (Gibco/BRL) as described previously (Wrana et al., 1992). Pools of cell colonies were maintained in the continuous presence of hygromycin. FlagMADR2 expression was induced by the overnight incubation of cells in medium containing 0.2% serum and 50 μM $ZnCl_2$.

Immunoprecipitations and Immunoblotting

Stably or transiently transfected cells were labeled for 2 hr with [^{32}P]phosphate as described previously (Attisano et al., 1996; Wrana et al., 1994). For stable cell lines, Zn^{2+}-induced monolayers were incubated in the presence or absence of 100 pM TGFβ (R and D Systems) in the last 15 min of the phosphate labeling. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 monoclonal antibody (IBI, Eastman Kodak), followed by adsorption to protein G–sepharose (Pharmacia). Immunoprecipitates were washed, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and visualized by autoradiography. For determination of MADR2 protein levels, lysates were prepared from cells treated in parallel with those subjected to in vivo phosphate labeling. Proteins from cell lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes. FlagMADR1 was detected using anti-FLAG M2 antibody (1:3000 dilution) and chemiluminesence as recommended by the manufacturer (ECL Kit, Amersham).

RH and YAC Contig Mapping

The following PCR primers specific for the 3′-UTR of MADR2 and DPC4 were designed using the Primer Version 3.0 program (Whitehead Institute–MIT Center for Genome Research): MADR2: forw. 5′-ACCAATCAAGTCCCATGAAA-3′; rev. 5′ TGATCGAGACCT CAAGTGCTG-3′; DPC4. UTR: forw. 5′ ATTGAAATTCACTTACACC GGG-3′; rev. 5′-AGCCATGCCTGACAAGTTCT-3′. The primers DCC. PCR2.1/2.2 and DCC. PCR36.1/36.2, which are specific for the most 5′ and 3′ exons, respectively, of DCC, are described in the GenBank Database. Information on all of the other primers shown in Figure 3 is available from the MIT Genome Center (http://www-genome.wi.mit.edu/) or is described by Hahn et al. (1996b). The conditions of PCR for all mapping experiments were: initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturing for 40 s at 94°C, annealing for 40 s at 55°C, and extension for 40 s at 72°C, 35 cycles. RH mapping experiments were carried out in duplicate using the Genebridge 4 panel (Walter et al., 1994; purchased from Research Genetics). The Whitehead Institute–MIT Center for Genome Research RH server was used to order the new STSs relative to framework markers. All of the protocols used for YAC manipulations have been described previously (Scherer and Tsui, 1991). MADR2 detected YACs C739a3, C749d11, C887e9, C929d7, and C940e10; DPC4 was positive for C747a6, C786c3, C917c8, C945b11, and C957b11; DCC. PCR2.1/2.2 detected 746h3, 787f7, 787g3, 818e6, 821b7, 838b4, 849d6, 945b11, and 956a9; and DCC. PCR36.1/36.2 identified 782g1, 787f7, 790d12, 821b7, 830g12, 838b4, 905e8, 945b11, and 966e5.

Mutational Analysis

cDNA was synthesized from tissue total RNA using Murine Moloney leukemia virus reverse transcriptase (Gibco–BRL) and random hexamers. Each SSCP PCR reaction was carried out in a 20 μl reaction composed of 1 × PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 0.01% gelatin), 1.6 mM MgCl2, 30 μM each dNTPs, 9 pmol of each forward and reverse primer, cDNA made from 25 ng of RNA, 1.5 μCi [^{33}P]-dATP (2000 Ci/mmol, NEN-DuPont), and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer). Regions 1 (nucleotides 279–542), 2 (nucleotides 778–1014), 3 (nucleotides 1182–1430), and 4 (nucleotides 953–1245) were amplified with the following primers: Region 1: 5′-AGATCAGTGGGATACAACAGG-3′ and 5′-GGCACTAATACTG GAGGCAA-3′ (264 bp); Region 2: 5′-AGCTTGGATTTACAGCC AGT-3′ and 5′-TAAGCGCACTCCTCTTCCTA-3′ (237 bp); Region 3: 5′-GGCTCAGTCTGTTAATCAGG-3′ and 5′-TTCCATGGGACTTGAT TGGT-3′ (249 bp); Region 4: 5′-TGTTAACCGAAATGCCACGG-3′ and 5′-TCTTATGGTGCACATTCTAGT-3′ (293 bp). Regions 1 and 2 were amplified simultaneously in the same reaction tube. Cycling conditions involved 35 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 20 s, using the 9600 Thermocycler (Perkin-Elmer). PCR product was mixed with 2 vol of denaturing dye (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, and 0.05% Xylene Cyanol FF), heat-denatured, and loaded on an SSCP gel (8% acrylamide: bis-acrylamide [40:1], 0.5 × Tris–borate–EDTA, and 10% glycerol). Electrophoresis was carried out at 16°C using a StrataTherm Cold temperature-controlled apparatus (Stratagene). Mutations were confirmed by direct sequencing of asymmetric PCR products.

Xenopus Embryonic Assays

The cDNAs for MADR2 and its mutant forms were subcloned into the CS2+ vector (Rupp et al., 1994), and plasmids were linearized with NotI prior to SP6 transcription of capped synthetic mRNA (using the mMessage Machine kit, Ambion Inc.). Transcripts from a linearized vector (pGEM 7) served as a negative control for mRNA injection. Each mRNA (1.0 ng) was injected into the animal pole of a two-cell blastula (0.5 ng per blastomere), and animal caps were excised at blastula stage 8 and cultured in vitro, or the embryos were allowed to develop intact to assay ectopic gastrulation movements. Proteins for Western blots were prepared by lysing animal caps (10 caps per 100 μl) directly in 2 × Laemmli gel loading buffer. RNA was prepared from animal caps and reverse transcriptase–PCR (RT–PCR) performed as described (Thomsen, 1996; Wilson and Melton, 1994) using a 25 μl reaction volume. A fraction (one-half) of a cap or 0.2 embryo equivalents were analyzed by RT–PCR, using 17 cycles of amplification for Ef1-α and 24 cycles for XBra. A fraction (one-fifth) of each sample was loaded on a 6% PAGE (0.5 × Tris–borate–EDTA) gel. Film (Kodak XAR) was exposed for 3 hr to detect Ef1-α and the embryo RT plus and minus lanes of Xbra. Xbra signals from animal caps were exposed for 6 hr.

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