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Novel interaction partners of the TPR/MET tyrosine kinase

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ABSTRACT

A large variety of biological processes is mediated by stimulation of the receptor tyrosine kinase MET. Screening a mouse embryo cDNA library, we were able to identify several novel, putative intracellular TPR/MET-substrates: SNAPIN, DCOHM, VAV-1, Sorting nexin 2, Death associated protein kinase 3, SMC-1, Centromeric protein C, and hTID-1. Interactions as identified by yeast two-hybrid analysis were validated in vitro and in vivo by mammalian two-hybrid studies, a far-western assay and coimmunoprecipitation. Participation in apoptosis-regulating mechanisms through interaction with DAPK-3 and cell cycle control via binding to nuclear proteins such as CENPC and SMC-1 are possible new aspects of intracellular MET signaling.

Key words: hepatocyte growth factor/scatter factor • signal transduction • embryonic development • tumorigenesis

Twenty years after its identification (1), the receptor tyrosine kinase MET, receptor for the hepatocyte growth factor/scatter factor (HGF/SF) (2, 3), is still fascinating because of its pivotal role in signal transduction, embryonic development, as well as in tumorigenesis. Binding of HGF, the sole target of the HGF-receptor/c-MET to the HGF-receptor leads to activation of its intrinsic kinase activity and tyrosine phosphorylation of a multifunctional docking site located at the C terminus. This mediates a high-affinity interaction for a set of components of the signaling complex containing a SRC homology 2 (SH2) domain, phosphotyrosine binding (PTB) domain or Met binding domain (MBD) (reviewed in (4)). Biological responses following MET activation include angiogenesis (5, 6), cellular motility (7), growth (8–10), invasion (11–13), apoptosis (14), and morphogenic differentiation (15, 16). Met mutations have been identified in patients with hereditary and sporadic papillary renal carcinoma, metastatic head and neck squamous cell carcinomas, and isolated cases of ovarian cancer and early-onset hepatocellular carcinoma (reviewed in (4)). Elevated MET and/or HGF/SF expression has been reported in a variety of human tumors and is associated with high tumor grade and poor prognosis (13). Recent studies revealed that MET-expression is switched on by low oxygen levels within the tumor, thereby causing a more aggressive and invasive phenotype of the respective cancers (17).

However, we are far from understanding how all these cellular processes can be transmitted through the activation of one single protein. Some of the key players of the intracellular MET-signaling complex are known, and most of them contain an SH2 domain, PTB, or MBD domain. These are GRB-2-SOS, phospholipase C-γ (PLCγ), the p85β subunit of phosphotidylinositol 3-kinase (PI 3-K), the SRC kinase, SHC, GAB1, STAT-3, and SHPTP2/SYP (reviewed in (18)). Indirect binding and phosphorylation of other proteins and activation of multiple pathways may further widen the spectrum of biological responses elicited by HGF/SF stimulation of cells (4, 19, 20).

It is a matter of general interest to know whether or not the common set of known MET-substrates is sufficient to explain the complex and pleiotropic effects after HGF stimulation. It has been discussed that quantitative (strength or duration of the signal) or qualitative (different substrates being recruited to MET depending on tissue-specific expression profiles) differences of intracellular signaling contribute to the plethora of biological consequences (21). Both models are not mutually exclusive. Recent publications indicate that the MET tyrosine kinase receptor can interact with other novel interaction partners such as a specific splice form of the hyaluron receptor CD44, β4-integrin, ezrin, the FAS receptor, semaphorin receptors, or E-cadherin, which may bring more light to this point of debate (22–26). The identification of eight novel intracellular interaction partners of MET as reported in this article strengthens the idea that specific proteins are responsible for the induction of specific cellular responses.

MATERIALS AND METHODS

Yeast two-hybrid assay

MATCHMAKER Two-Hybrid System 2 (Clontech) was used with pGBT9-TPR/MET serving as bait. The cDNA encoding for the constitutively active form of the MET receptor was amplified by PCR using the complete *tpr/met* cDNA as template and the following oligonucleotides (BamHI-sites underlined): 5′-CGCGGATCCTAATGGCGGCGGTGTTGCA-3′ (for) and 5′-CGCGGATCCCTAGCACTATGATGTCTCCC-3′ (rev). The PCR product was digested with BamHI, purified and subcloned into pGBT9. pGBT9-*tpr/met* was transformed into the *S. cerevisiae* strain HY (27), followed by sequential introduction of plasmid DNA of a rat embryo cDNA library in pGADGH yeast expression vector (Clontech, Palo Alto, CA). Positive colonies were picked and assayed for LacZ activity using a filter β-galactosidase assay. LacZ-positive cDNA clones were rescued from the selected yeast transformants and sequenced by the dideoxy terminator cycle sequencing method using a CycleReader Auto DNA sequencing kit (MBI Fermentas, St. Leon-Rot, Germany) and labeled GADGH primers, THGAD5′IRD700 (5′-CGATGATGAAGATACCCCACC-3′) and THGAD3′IRD800 (5′-CACGATGCACAGTTGAAGTG-3′) on a Li-Cor Readir4200 sequencer (MWG Biotech, Ebersburg, Germany). Sequences were analyzed by BLAST for sequence homology and conserved domains by BLASTN 2.2.1 (NCBI database).

Yeast colony hybridization was performed using β-glucuronidase (Sigma-Aldrich, St. Louis, MO) to break cell walls, and colonies were lifted onto Nytran 13N nylon membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) as recommended by the manufacturer (Yeast protocol handbook, Clontech). Filters were hybridized in 3 × SSC (0.45 M NaCl, 0.045 M sodium

citrate), 5 × Denhardt's, 200 μg/ml denatured salmon sperm DNA, 1% SDS and 10% dextran sulfate at 63°C for 16 h with a hexalabeled *Grb-2* insert probe (HexaLabel DNA Labeling Kit, MBI Fermentas) with 2 μg/ml plasmid DNA as a competitor to avoid cross-hybridization with yeast binding- and activating-domain containing plasmid DNAs. Subsequently, the filters were extensively washed in 3 × SSC, 0.1% SDS at 63°C, followed by a wash at moderate or high stringency. Filters were exposed to Kodak X-Omat DS film at −70°C with Ilford intensifier screens.

Eukaryotic two-hybrid system

The CHECKMATE mammalian Two-Hybrid System was purchased from Promega. cDNAs of putative interaction partners were cloned into the pACT vector, the PCR being performed using pGADGH-interaction partner-DNA as template, sense primer 5′-CGGGATCCGTCGACTTGACGCGTTCCCGGGCTGCAG-3′ and antisense primer 5′-GCTCTAGAGCGGCCGCGGTACCGGGCCCCCCCTCGA-3′. PCR-products were cloned into pGemTeasy, checked by sequencing, digested with NotI and SalI, and cloned into pACT. *Met* and *tpr/met* were cloned into the pBind vector via BamHI sites. For this purpose, *met* was PCR-amplified using a pGBT9-*met* as a template and the following primers (BamHI-sites underlined): 5′-CGCGGATCCTCGATCAGTTTCCTAATTCATCTC-3′ (for) and 5′-CGCGGATCCCTATGATGTCTCCCAGAAGG-3′ (rev). Amplification of *tpr/met* was carried out with pGBT9-*tpr/met* as template and the primers 5′-CGCGGATCCTCGCGGCGGTGTTGCAGCAAG-3′ (for) and 5′-CGGGGATCCCTAGCACTATGATGTCTCCC-3′ (rev). Cotransfections for the eukaryotic two-hybrid assay were performed using 24-well-plates with 80.000 HEK-293 cells/well and GeneJuice transfection reagent. A pBind-receptor:pACT-substrate:pG5luc ratio of 2:1:1 was used. Transfection efficiency and interaction of receptor and substrate were checked using Dual-Luciferase Reporter Assay System provided by Promega. Cell lysis and detection of Renilla- and Firefly-Luciferase activity were carried out as recommended by the manufacturer with a Lucy 2 luminometer (Anthos Mikrosysteme, Krefeld, Germany).

Affinity purification and in vitro autophosphorylation of TPR/MET protein

TPR/MET polypeptide was purified using Ni2+-chelat affinity chromatography. To this end, *tpr/met* was cut out of pGBT9-*tpr/met* with BamHI and cloned into pcDNAHisMaxA vector (Invitrogen, Carlsbad, CA). HEK-293 cells were transiently transfected with pcDNAHisMaxA-*tpr/met* using calcium phosphate (28). Purification and in vitro labeling was performed as described by (29).

GST fusion proteins and far-western analysis

cDNAs of potential *tpr/met* interaction partners were expressed in *E. coli* as GST-fusion proteins. cDNA was cloned from pGADGH into pGEX-4T3 (Amersham) using EcoRI and XhoI restriction sites. cDNA of *CenpC* was partially digested (15 μg DNA, 10 U XhoI, 200 U EcoRI) because of an internal XhoI restriction site. Far Western analysis was essentially performed as described previously by us (29). Successful induction of GST fusion proteins was investigated by incubating duplicate filters with anti-GST monoclonal antibodies (Santa Cruz).

Generation of TPR/MET K241A and TPR ΔMET using site directed mutagenesis

An inactive mutated kinase negative form of TPR/MET and TPR alone was also used for coimmunoprecipitation experiments. The amino acid essential for binding of ATP and thus autophosphorylation of the MET receptor, lysine241 in TPR/MET, was changed by site-directed mutagenesis into alanine. Mutagenesis was carried out using pGemTeasy–tpr/met as template, 5′-TGGCAAGAAAATTCACTGTGCTGTGGCATCCTTGAA-3′ as mutagenic primer (mutation underlined) and 5′-GTGACTGGTGAGGCCTCAACCAAGTC-3′ (ScaI to StuI) as selection primer in the Transformer site-directed mutagenesis kit (Clontech). *Tpr/metK241A* was cloned via NotI digestion from pGemTeasy into pMT2SM-3xHA (eukaryotic expression vector). *Tpr*Δ*met* was generated by site-directed mutagenesis using pMT2SM-3HA-*tpr/met* as template and the primer TPR/ENDMETdel 5′-CTCAAGAACTTGAATACTTAACATAGTGCTAGGAATTCGGGG-3′ in the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). Correct deletion of the *met* portion was verified by sequencing.

Coimmunoprecipitation

For coimmunoprecipitation we created a 3xHA tagged eukaryotic expression vector: pMT2SM3xHA. Two oligonucleotides pMT2SM3xHAfor 5′ PHO-GCCACCATGGCACCATACGATGTTCCAGATTACGCTTATCCCTATGACGTGCCCGAC TATGCCTACCCTTACGATGTCCCTGATTACGCGGG 3′ and pMT2SM3xHArev 5′ PHO-TCGACCCGCGTAATCAGGGACATCGTAAGGGTAGGCATAGTCGGGCACGTCATAGG GATAAGCGTAATCTGGAACATCGTATGGGTATGCCATGGTGGCTGCA 3′ were annealed and cloned into Sal I and Pst I digested pMT2SM expression vector. Using the vector's Sal I and Eco RI restriction site we were able to clone our receptor constructs *tpr/met* and *tpr/metK241A* into this new vector. Interaction partners containing complete cDNA inserts (*Snapin* and *DcoHM*) or part of the complete cDNA (*Vav-1*) were cloned into pcDNA/HisMax B (Invitrogen) via Eco RI and Xho I restriction sites. For SNX-2, we used the complete coding sequence attached to a myc tag (kindly provided by Dr. Carol Haft, NICHD-CBMB, NIH, USA). Complete cDNA inserts of the other interaction partners cloned into pcDNA/HisMaxB or C eukaryotic expression vectors were constructed as follows. DAPK-3 was amplified from rat Dlk cDNA (kindly provided by Dr. Donat Kögel, Dept. Experimentelle Neurochirurgie im ZBC, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt) using primers Dlkfor 5′-CCGGAATTCGTCCACGTTCAGGCAG-3′ and Dlkrev 5′-CCGCTCGAGCTAGCGCACGCCGCACTC-3′ by Pfu polymerase and cloned into pcDNAHisMaxB. Complete rat *Grb-2* cDNA was amplified from DNA of the rat embryo cDNA library (see above) using primers ratASHfor 5′-CCGGAATTCCGAAGCCATCGCCAAATATGAC-3′ and ratASHrev 5′-CCGCTCGAGTTAGACGTTCCGGTTCACTGG-3′ by Pfu polymerase and cloned into pcDNAHisMaxB. Complete *Smc-1* cDNA was obtained from the Human cDNA Bank Section of the Kazusa DNA Research Institute (KIAA0178). A BamHI restriction site was inserted 5′ to the open reading frame by amplifying the 5′ part of the *Smc-1* cDNA using primers SMC1Bamfor 5′-GCGGGATCCGGGTTCCTGAAACTGATTGAG-3′ and SMC1rev 5′-GAGCCCGTACTACCTCATCC-3′ by Pfu polymerase. A BamHI/KpnI fragment from the PCR product and a KpnI/EcoRI fragment from the cDNA were ligated together into pcDNAHisMaxC. Complete *hTid-1* cDNA was obtained from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (clone IRALp962O1430Q2). An EcoRI restriction site was introduced 5′ to the open reading frame by amplifying the 5′ part of the *hTid-1* cDNA using primers

hTidEcoRIfor 5′-CGGAATTCGCTGCGCGGTGCTCCAG-3′ and hTidrev 5′-CCCCCTTTGCAGCTTGATTG-3′ by Pfu polymerase. An EcoRI/BglII fragment from the PCR product and a BglII/XhoI fragment of the cDNA were ligated together into pcDNA/HisMaxC. Complete *Cenpc* cDNA was obtained from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (clone IRAKp961E1782Q2). An EcoRI restriction site was introduced upstream of the open reading frame by amplifying the 5′ part of the *Cenpc* cDNA using primers CENPCEcoRIfor 5′-CGGAATTCGCTGCGTCCGGTCTGGATC-3′ and CENPCrev 5′-CTTCATCAGCTTCACTGTGAT-3′ by Pfu polymerase. An EcoRI/SalI fragment from the PCR product and a SalI/EcoRI fragment from the cDNA were ligated together into pcDNAHisMaxC. At first, all PCR fragments were cloned into T-vector and verified by sequencing. Correct cloning into pcDNAHisMax was also verified by restriction enzyme digestion and sequencing. Because of the fact that the protein expressed from complete *Snapin* cDNA has a similar molecular weight as the light chain of the precipitating antibody and no suitable HRP-labeled anti-Xpress antibodies are available, *Snapin* cDNA was excised from pGADGH and cloned into pMT2SM3xFLAG using KpnI and XbaI restriction sites. For coimmunoprecipitation HEK-293 cells were transiently cotransfected by DNA/calciumphosphate precipitate with a receptor:substrate-ratio of 1.5:2.5. 72 h after transfection cells were lysed in RIPA buffer (1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 400 mM NaCl, 50 mM Tris/HCl pH 7.2) with 1 × protease inhibitor cocktail EDTA-free (Roche), 1 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/ml aprotonin. Cell lysates were diluted with an equal amount of PBS and precleared with Protein A Sepharose CL-4B (Amersham Biosciences) for 30 min at 4°C. Supernatants were incubated with 2 μg anti-HA antibody (Sigma) and with gentle rotation agitated at 4°C overnight. The next day Protein A Sepharose was added to the lysates followed by gentle rotation at 4°C for 5 h. After centrifugation at 4°C, Sepharose beads were washed 4 times with washing buffer (50% RIPA buffer/50% PBS with protease inhibitors) and finally dissolved in 2× SDS sample buffer. Further analysis was carried out by SDS-PAGE and Western blot. Detection of the different interaction partners was performed with anti-Xpress (Invitrogen), anti-myc antibody (Babco), or HRP-labeled anti-Flag antibody (Sigma), following the manufacturer's protocol.

RESULTS

Isolation of TPR/MET binding proteins in a yeast two-hybrid screen

To gain further insight into the intracellular signaling network underlying the diverse processes induced by activation of the MET-receptor, we initially used a yeast two-hybrid assay. The oncogenic TPR/MET fusion protein was used as bait in a rat embryo cDNA library screening. *Tpr* sequences encode a leucine-zipper dimerization motif, leading to constitutive dimerization, and thus, tyrosine-phosphorylation of the TPR/MET fusion protein (30, 31). After screening 9×106 transformants, 290 His-positive clones were selected and tested by qualitative X-Gal-Assay. 86 yeast colonies were found to be positive in the qualitative β-galactosidase assay for LacZ reporter expression, 31 of them showed weak and 55 strong β-galactosidase activity. Because GRB-2 is known as a strong MET-interaction partner, we used a yeast colony hybridization procedure to analyze colonies showing a strong β-galactosidase activity for the presence of *Grb-2* sequences. Twenty-five of the 55 tested yeast colonies were positive for *Grb-2* sequences using a radioactively labeled *Grb-2* probe. Activating domain plasmids with inserts

of the remaining (*Grb-2*-negative) colonies in combination with an empty bait vector or our original bait (TPR/MET) were retransformed into HY yeast cells and tested in the β-galactosidase assay. Most GRB-2-negative His-positive transformants formed blue colonies only in combination with our original bait (TPR/MET). Activating domain plasmids with insert that were positive in combination with the empty bait vector and mostly code for transcription factors that can activate the LacZ reporter solely (e.g., goosecoid) were discarded.

Specificity of TPR vs. MET interaction

To determine the specificity of the interaction, the strongly positive GADGH-plasmids were cotransformed into the HY yeast strain together with a bait plasmid containing the complete *tpr/met* construct, the *met* part (cytoplasmic portion of *met*) or the *tpr* part, respectively, cloned into the pGBT9 bait vector. Colonies were selected and analyzed by X-Gal-Assay for LacZ reporter expression. All 30 GRB-2-negative clones showed moderate to high interaction with TPR/MET and low to high interaction with the cytoplasmic part of the MET receptor alone. One out of 30 clones showed interaction with the TPR portion of TPR/MET, which turned out as cDNA coding for Laminin B1. Searching for MET-interacting proteins, this TPR-interacting clone was not further investigated. The other putative interaction partners did not interact with the TPR protein.

Sequence analysis of the interaction partners

Plasmid DNAs of the strongly positive interaction partners were sequenced and compared with the GenBank database. 13 clones suspected to be false positives were not investigated further (e.g., mitochondrial cytochrome oxidase, ribosomal protein B20) (32). Putative physiological interaction partners of the TPR/MET tyrosine kinase as identified by our yeast two-hybrid analyses are shown in Table 1. Protein sequences of putative TPR/MET interaction partners and that part of the protein present in the isolated "prey" plasmid are depicted in Fig. 1. Four interaction partners were detected only once in our screen (SNAPIN, VAV-1, SNX-2, and SMC-1), DAPK-3, CENPC and DCOHM could be isolated twice and mTID-1 was isolated from five independent clones, respectively.

Verification of the interaction between TPR/MET and the identified partners by mammalian two-hybrid analysis

In higher, eukaryotic organisms, most proteins undergo extensive post-translational modifications, which are essential for their binding affinities and functions. Some of the modifications (e.g., tyrosine phosphorylation, glycosylation, and disulfide bond formation) are underrepresented in the yeast two-hybrid system (33). To verify that the interactions between TPR/MET and the new putative substrates discovered in yeast can also take place in a mammalian environment, we performed a mammalian two-hybrid assay. To learn more about binding affinities of putative substrates to MET and the oncogenic fusion protein TPR/MET, we performed mammalian two-hybrid studies with both constructs serving as "bait". Activation of the MET-part alone is ensured through dimerization of the GAL4 DNA binding domain of the bait vector. HEK-293 cells were cotransfected with *tpr/met* and *met*-constructs subcloned into pBind, the various interaction partners subcloned into pACT vector, and the reporter vector pG5luc (reporter gene: firefly luciferase). pBind contains and expresses the *Renilla reniformis*

luciferase gene that allows normalization of transfection efficiency. Table 2 shows that the firefly luciferase activity of the reporter is significantly higher with all interaction partners than the empty bait vector used as a control (set at 100%). Binding to MET is stronger than to TPR/MET for most interaction partners except for SMC-1, which shows a stronger affinity to TPR/MET. GRB-2, a well-known strong interaction partner of MET, served as a positive control.

Far Western analysis supports direct interaction between TPR/MET and its partners

To confirm our results from the two-hybrid assays, we examined the interactions by Far Western analysis. Insert DNAs of the interaction partners were cut out of the pGADGH plasmid and recloned in frame into a GST fusion protein vector. Respective plasmid DNAs were transformed into *E. coli* cells and protein synthesis induced by IPTG induction. Bacterial lysates induced and noninduced GST proteins were separated on gels and Western blotted. Filters were incubated with anti-GST antibodies or incubated with [32P]-labeled and purified HA-tagged TPR/MET protein, respectively. The oncogenic variant of the MET receptor was used to ensure constitutive activation of the receptor tyrosine kinase. Successful induction of the GST-fusion proteins on duplicate filters, incubated with GST-monoclonal antibody is demonstrated (Fig. 2, upper panel). Incubation of the Western blots with the labeled TPR/MET protein shows a strong interaction with DAPK-3, SMC-1, and GRB-2 (Fig. 2, lower panel). Moderate binding was observed with CENPC and mTID-1, and a weak interaction was demonstrated with SNAPIN, DCOHM, VAV-1 and SNX-2. This weak binding could be due to the absence of adaptor proteins needed for TPR/MET binding or a strong tertiary structure of these interaction proteins that allow TPR/MET binding, but does not completely resist the denaturing conditions of SDS-electrophoresis, blotting and incubation in Simon buffer.

In vivo interaction between TPR/MET and its interaction partners

Coimmunoprecipitation analysis represents one of the most rigorous methods to demonstrate protein–protein interaction in vivo. Therefore, complete cDNAs or cDNA fragments of the various interaction partners were cloned into the eukaryotic expression vector pcDNA/HisMax B or C exhibiting a 6x His-tag and an additional Xpress-tag at the multiple cloning site's NH2-terminus. *Tpr/met*, *tpr/met*K241A, and *tpr*Δ*met*, were cloned into pMT2SM3xHA eukaryotic expression vector, containing a 3xHA-tag. TPR/MET K241A is a kinase-negative variant of TPR/MET because of its inability to bind ATP. TPRΔMET contains the TPR portion solely. Coimmunoprecipitation studies were performed with extracts of transiently cotransfected HEK-293 cells and anti-HA antibodies to coimmunoprecipitate the transiently transfected and thus overexpressed receptor constructs specifically. Total cell lysate was checked for proper expression of TPR/MET and the analyzed interaction partners by immunoblotting with anti-HA, anti-Xpress or HRP-labeled anti-FLAG antibodies, respectively (Fig. 3*A*, *B*). Anti-HA antibodies were able to coimmunoprecipitate TPR/MET and the following interaction partners: DCOHM, VAV-1, SNX-2 and hTID-1. DCOHM, SNX-2, and hTID-1 were also coimmunoprecipitated with the kinase inactive mutant TPR/MET K241A (Fig. 3*C*). It seems that these interaction partners can bind to TPR/MET independently of its phosphorylation status. SNAPIN, DAPK-3 and SMC-1 could only be coimmunoprecipitated with TPR/MET K241A and not with wild-type TPR/MET. None of the interaction partners was coimmunoprecipitated with TPRΔMET, implying that all partners bind to the MET portion of the TPR/MET tyrosine kinase and not to TPR. We were not able to demonstrate convincingly the coimmunoprecipitation of CENPC.

Coimmunoprecipitation of GRB-2 (the positive control) was positive with TPR/MET and not with TPR/MET K241A as expected.

DISCUSSION

Despite enormous advances in our understanding of receptor tyrosine kinase signaling, we still face the intriguing question of how one receptor, in our case the MET proto-oncogene, can evoke highly different cellular responses by recruiting a rather limited repertoire of signaling molecules. One might propose quantitative differences in receptor stimulation, cross-talk between the signaling cascades of various RTKs, or qualitative aspects, that is, distinct interaction partners being responsible for specific cellular responses.

The identification of several novel putative intracellular interaction partners of the TPR/MET tyrosine kinase strongly supports the idea that specific signaling molecules can be ascribed for individual aspects of the biological response of the HGF-receptor, c-MET. In this paper, we report the identification of a set of different proteins that interact with the TPR/MET protein by application of the yeast two-hybrid system. Among these proteins is GRB-2, a known interaction partner of MET, which confirms the suitability of our approach. Other known interaction partners of MET (e.g., PLC-γ, STAT-3, SHC) were not detected in our screen, which could be attributed to the fact that each cDNA library (we used a rat embryo library), has only a limited capacity for detecting interaction partners because of tissue distribution and sequence divergence between species.

The putative TPR/MET substrates detected by yeast two-hybrid became subject to a number of additional investigations clarifying different aspects of their putative binding to the TPR/MET tyrosine kinase. Mammalian two-hybrid studies confirmed the yeast data and proved that the identified interactions are also recognized in a eukaryotic context. Far Western analysis revealed that most of the interaction partners are able to bind TPR/MET directly in vitro. Finally, coimmunoprecipitations provided strong evidence that SNAPIN, DCOHM, VAV-1, SNX-2, DAPK-3, SMC-1, and hTID-1 can interact with the TPR/MET tyrosine kinase in eukaryotic cells under almost physiological conditions. Table 3 gives an overview of all different experiments performed with the novel interaction partners identified in this study. Although Far Western analysis and coimmunoprecipitation were only performed with TPR/MET and not with MET, all data together suggest that these interaction partners would likely also bind to the HGF-receptor, c-MET. Interaction of CENPC with TPR/MET could not be proven directly by coimmunoprecipitation. This could be due to incorrect folding or insolubility in the lysis buffer. Coimmunoprecipitations provided us with additional information concerning the dependency of identified interactions on the phosphorylation status, that is, the kinase activity of the TPR/MET protein.

SNAPIN and SNX-2 interact with TPR/MET

SNAPIN was originally identified as a synaptic vesicle membrane protein which associates with the SNARE complex through its direct interaction with SNAP-25 (34). SNAPIN is ubiquitously expressed and interacts with SNAP-23 in nonneuronal tissue (35). SNAPIN showed direct interaction with TPR/MET in the Far Western assay, was weakly positive in the mammalian two-hybrid, but could convincingly be coimmunoprecipitated with TPR/MET K241A, not with

TPR/MET. In the light of our studies, it is highly interesting that SNAP-25 interacts with HRS (hepatocyte growth factor regulated tyrosine kinase substrate) (36), a protein involved in the degradation of MET through internalization and sorting to lysosomes (37), which in turn serves as a binding partner of Sorting nexin 1 (SNX-1). SNX-1 again is highly homologous to and forms a heterodimer with Sorting nexin 2 (SNX-2). In fact, we identified SNX-2 as a novel interaction partner of TPR/MET. Our results therefore add to a more integrated understanding of the complex web of interactions involved in the degradation of receptor tyrosine kinases. Future studies will show whether a multiprotein complex of SNARE-25, SNAPIN, MET, SNX-2, SNX-1 and HRS exists or if single proteins compete for MET binding and thus induce distinct cellular effects.

Death-associated protein kinase 3 (DAPK-3)

In addition to its mitogenic activity, HGF has cytotoxic activity which suppresses the growth of several tumor cell lines, inhibits the growth of hepatocarcinogenesis in vivo and in a transgenic mouse model coexpressing c-MYC and HGF. Besides the activation of caspase-3, little is known about the signaling pathway for the cytotoxic activity of HGF [(38) and references herein; (39)]. Recently, the direct interaction and sequestration of the death receptor FAS in hepatocytes by the MET receptor has been reported as a mechanism of cell survival (24). The DAP (Death Associated Protein) kinase family represents a novel subfamily of proapoptotic serine/threonine kinases. All five DAP kinase family members identified to date are ubiquitously expressed in various tissues and are involved in both extrinsic and intrinsic pathways of apoptosis. Their sequence homology is largely restricted to the N-terminal kinase domain. In contrast, the adjacent C-terminal regions are diverse and link individual family members to specific signal transduction pathways. DAPK-3/DLK (synonym: Death-like kinase, DLK) carries a functional C-terminal leucine zipper motif, which mediates both homodimerization and interaction with several non-DAPK proteins. DAPK-3 is naturally localized in the nucleus, but binding of PAR-4 leads to a switch in subcellular localization from the nucleus to the cytoplasm and colocalization with actin filaments (40). In accordance with the data mentioned above, the TPR/MET screen led to the isolation of the C-terminal end of DAPK-3. Moreover, this protein is strongly positive in the Far Western analysis and showed a noticeable induction of the luciferase activity in the eukaryotic two-hybrid assay. Interestingly, our coimmunoprecipitations demonstrated that DAPK-3 preferably interacts with the inactive form of the TPR/MET tyrosine kinase in vivo. Thus, DAPK-3 resembles FAP68, which interacts selectively with the nonphosphorylated MET receptor (41). It will be of special interest to investigate the implications of the interaction between MET and DAPK-3 in the regulation of apoptosis.

SMC-1 and possibly CENPC bind to TPR/MET

Structural maintenance of chromosomes (SMC) family proteins have attracted much attention for their unique protein structure and critical roles in mitotic chromosome organization. In human cells SMC-1 forms a heterodimer, with SMC-3 being part of the cohesin-complex which is required for sister chromatid cohesion and thus a prerequisite to accurate segregation of chromosomes (reviewed in (42)). Centromeric protein C is localized toward the outer centromere and might have a role in the connection between centromeric chromatin and the kinetochore (reviewed in (43)). The identification of both SMC-1 and CENPC as novel interaction partners of TPR/MET suggests that there could be a link between SMC-1 and CENPC as well. This idea

is favored by the fact that MIF2, the yeast homologue of CENPC is known to interact with SCC-1, one of the four structure-giving molecules of the cohesin-complex. An important question to be answered is where the identified interactions take place. It is surprising that nuclear proteins are identified as substrates of a membrane-spanning receptor. But on one hand, we know that HCP-4, a CENPC-like protein is localized in the cytoplasm during the interphase (44). On the other hand, a putative nuclear translocation of plasma membrane-associated receptor tyrosine kinases has recently been discussed (45).

Other proteins interacting with TPR/MET

VAV-1, DCOHM, and hTID-1 could also be identified as novel interaction partners of the TPR/MET oncogene. All these provide additional, interesting features to be further investigated in the new light of MET association. Binding of VAV-1 to TPR/MET seems plausible—it is the only novel interaction partner containing a SH2-domain, and many VAV proteins are known to interact by means of their SH2 domains with several receptor tyrosine kinases (46).

DcoHM is a gene that has recently been identified as MIRK binding protein by yeast two-hybrid analysis (47). MIRK/DYRK1B is an arginine-directed serine/threonine protein kinase that is expressed at low levels in most normal tissues but at elevated levels in many tumor cell lines and in normal skeletal muscle. DCOHM (dimerization cofactor of hepatocyte nuclear factor 1 α (HNF1α) from muscle), a novel gene of the DCOH family stabilizes HNF1α as a dimer and enhances its transcriptional activity on the β-fibrinogen promoter reporter, like DCOH. MIRK coimmunoprecipitated with the MAPK kinase MKK3, an upstream activator of p38. It has been suggested that MIRK may function as an HNF1α transcriptional activator in response to an MKK3-mediated stress signal, and the selective expression of DCOH could restrict the MIRK response to carcinoma cells (47).

hTID-1 is a member of the DnaJ family that functions as a molecular chaperone and interacts with Hsp70 in the presence of IFN-γ (48). Two splice variants of the human *Tid-1* gene, *hTid-1S* and *hTid-1L*, were identified. They have opposing effects on apoptosis in response to tumor necrosis factor-α or mitomycin C (49). Interaction of mTID-1 with RAS GTPase-activating Protein (GAP) in a phosphorylation-independent fashion is known (50). mTID-1 sequences were isolated from five independent clones as interaction partners of the TPR/MET tyrosine kinase in the yeast two-hybrid screen. The interaction between mTID-1 and MET probably provides new hints at the apoptosis-modulating potential of MET.

In the age of proteomics, biological research tries to face the study of the function and interaction of all expressed proteins of a cell. Tremendous progress has been made in generating large-scale data sets for protein–protein interactions (51). But every novel substrate as identified by yeast two-hybrid has to be validated using additional, more rigorous experiments—no matter whether it has been gained from a large-scale approach or a classical two-hybrid screen. And still it is mostly the information gained from the study of individual proteins or small protein sets that forms the basis of continuing, functional studies. The identification of several novel putative interaction partners of the TPR/MET tyrosine kinase provides such information. It shows that the intracellular signaling network associated with MET is by far more complex than previously supposed. Our data yield a multitude of exciting questions that deserve further investigation.

Functional studies facing these questions will contribute to a more integrative understanding of intracellular signaling following stimulation of the MET receptor.

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Table 1

TPR/MET-interacting proteins detected by yeast two-hybrid studies

Name	Known functions
SNAPIN	Enriched in neurons, associated with the SNARE complex; role in neurotransmitter release
DCOHM	Dimerization cofactor of hepatocyte nuclear factor 1 from muscle
VAV-1	Guanine-nucleotide exchange factor of the Rho/Rac family; major expression in hematopoetic cells
Sorting nexin 2 (SNX-2)	Member of the Sorting nexin family; role in protein transport from and to the endosome
Death associated protein kinase 3 (DAPK-3)	Serine/threonine kinase with apoptosis-regulating functions
Structural maintenance of chromosomes 1 (SMC-1)	Part of the cohesin complex, of major importance to chromosome condensation, loss of sister chromatid cohesion, DNA repair, and recombination and gene dosage compensation
Centromeric protein C (CENPC)	Part of the centromere, of importance to the correct association of the kinetochore complex and metaphase–anaphase transition
mTID-1	A mitochondrial-located DnaJ chaperone protein with modulating effects on apoptotic signals
GRB-2	Adaptor protein and known interaction partner of MET

Table 2

Results of the mammalian two-hybrid analysis

	SNAPIN	DCOHM	VAV-1	SNX-2	DAPK-3	SMC-1	CENPC	mTID-1	GRB-2
Empty vector set at	100%	100%	100%	100%	100%	100%	100%	100%	100%
TPR/MET	596%	1220%	340%	10,023%	1422%	3287%	1343%	616%	9158%
MET	1407%	4149%	2281%	22,896%	2556%	1686%	4837%	1603%	31,651%

Reporter (Firefly luciferase) activity, normalized for transfection efficiency.

Table 3

Overview of all novel interaction partners identified in this study

Method	Construct	SNAPIN	DCOHM	VAV-1	SNX-2	DAPK-3	SMC-1	GRB-2	CENPC	m/hTID-1
Yeast two-hybrid	MET	+	++	++	++	+	+	++	++	++
Far Western	TPR/MET	+	+	+	+	++	++	++	++	++
Mammalian two-hybrid	MET	+	++	+	++	+	+	++	++	+
Co-IP	TPR/MET	–	++	+	++	–	–	++	?	++
	TPR/MET K241A	++	+	–	+	+	+	–	?	+++

–, negative; +, slightly positive; ++, positive; +++, strongly positive; ?, not detectable because of the insolubility of the protein in lysis buffer.

Fig. 1

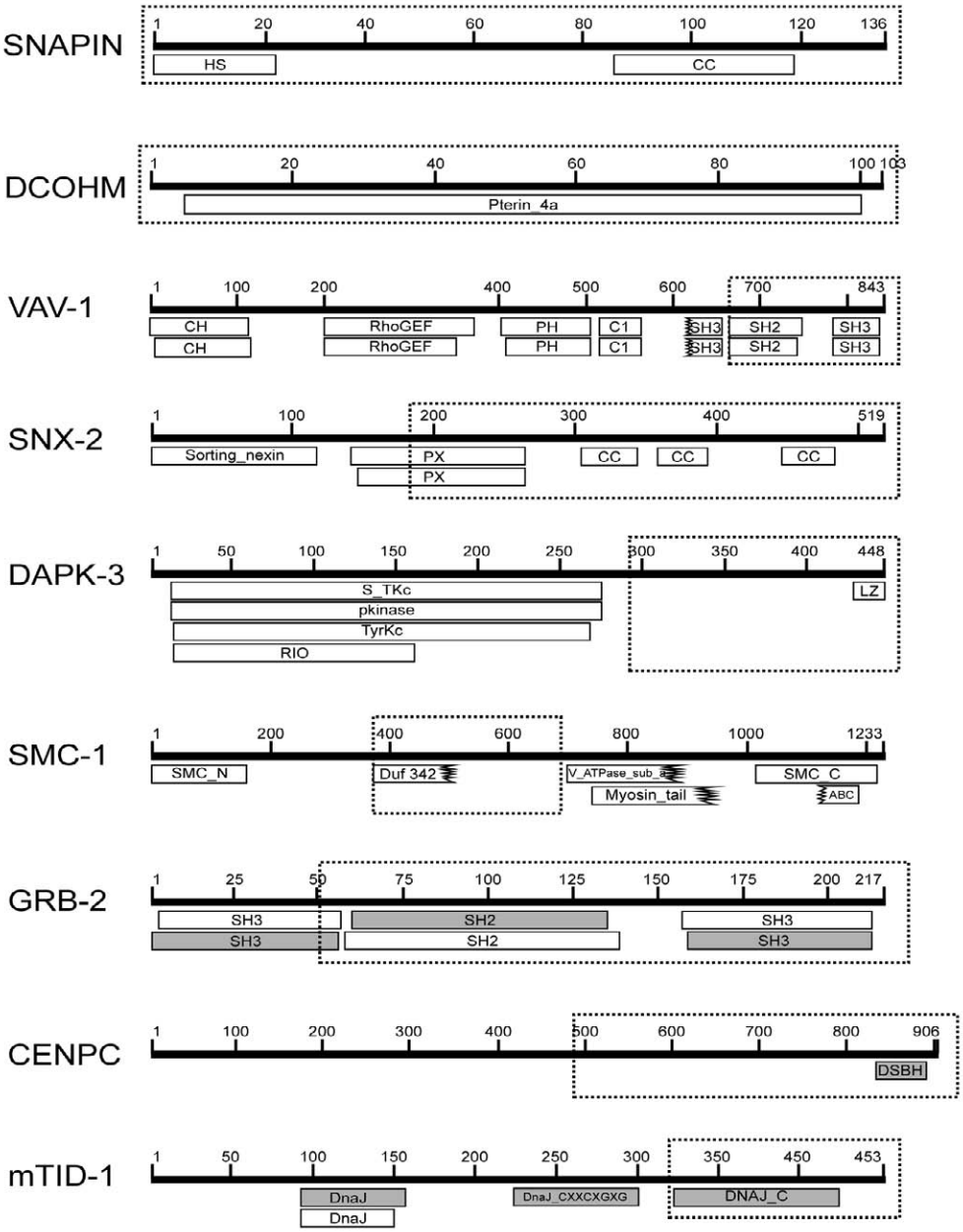

Figure 1. Sequence analysis of two-hybrid positive clones showing homology to known genes in the database. Plasmid DNAs of the TPR/MET-positive interaction partners were sequenced and compared with the GenBank database. Proteins with their conserved domains as revealed from a blast search at the NCBI database for conserved domains are depicted (HS, hydrophobic segment; CC, coiled coil; Pterin_4a, Pterin-4-α-carbinolamine dehydratase; CH, calponin homology domain; RhoGEF, RhoGEF domain; PH, Pleckstrin homology domain; C1, Protein kinase C conserved 1 (C1) domains (Cyteine-rich domains); SH3, Src homology 3 domains; SH2, Src homology 2 domains; Sorting_nexin, Sorting nexin, N-terminal domain; PX, PX domain; S_Tkc, Serine/threonine protein kinases, catalytic domain; pkinase, Protein kinase domain; Tyrkc, Tyrosine kinase, catalytic domain; RIO, RIO-like kinase; LZ, Leucine zipper; SMC_N, RecF/RecN/SMC N-terminal domain of SMC proteins; DUF342, protein of unknown function (DUF342); V_ATPase_sub_a, V-type ATPase 116 kDa subunit family; Myosin_tail, Myosin tail; SMC_C, SMC family, C-terminal domain; DSBH, double-strand β helix domain; DnaJ, DnaJ domain; DNAJ_C, DnaJ C domain; DnaJ_CXXCXGXG, DnaJ central domain (4 repeats). Protein sequences present in our TPR/MET-interaction partners are indicated by a dotted box.

Fig. 2

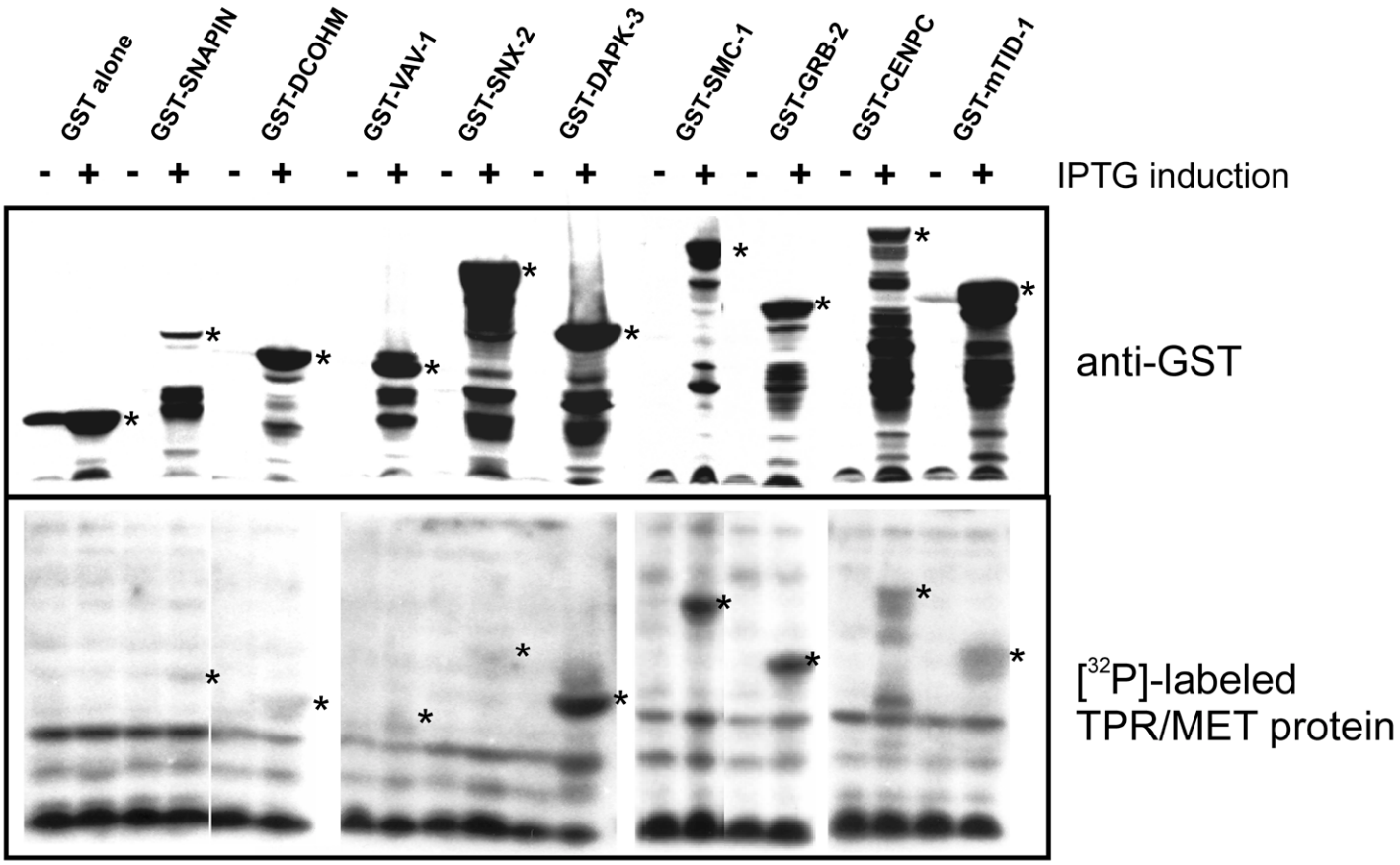

Figure 2. Far Western analyses of GST-fusion proteins of the interaction partners with [32P]-labeled TPR/MET protein. Bacteria containing the indicated GST-interaction partner plasmid constructs were induced with IPTG (+) or not induced (–). Cell lysates were analyzed by SDS-PAGE and Western blotting. Successful induction of GST fusion proteins was investigated by incubating duplicate filters with anti-GST monoclonal antibodies (***upper panel***). Because of protein degradation in bacteria, most lanes show more than one band. The largest band (marked by an asterisk) corresponds to the mature protein. Filters were incubated with [32P]-labeled TPR/MET protein, washed and exposed in order to detect TPR/MET binding to its various interaction partners (***lower panel***). Bound proteins are marked by a black or white asterisk.

Fig. 3

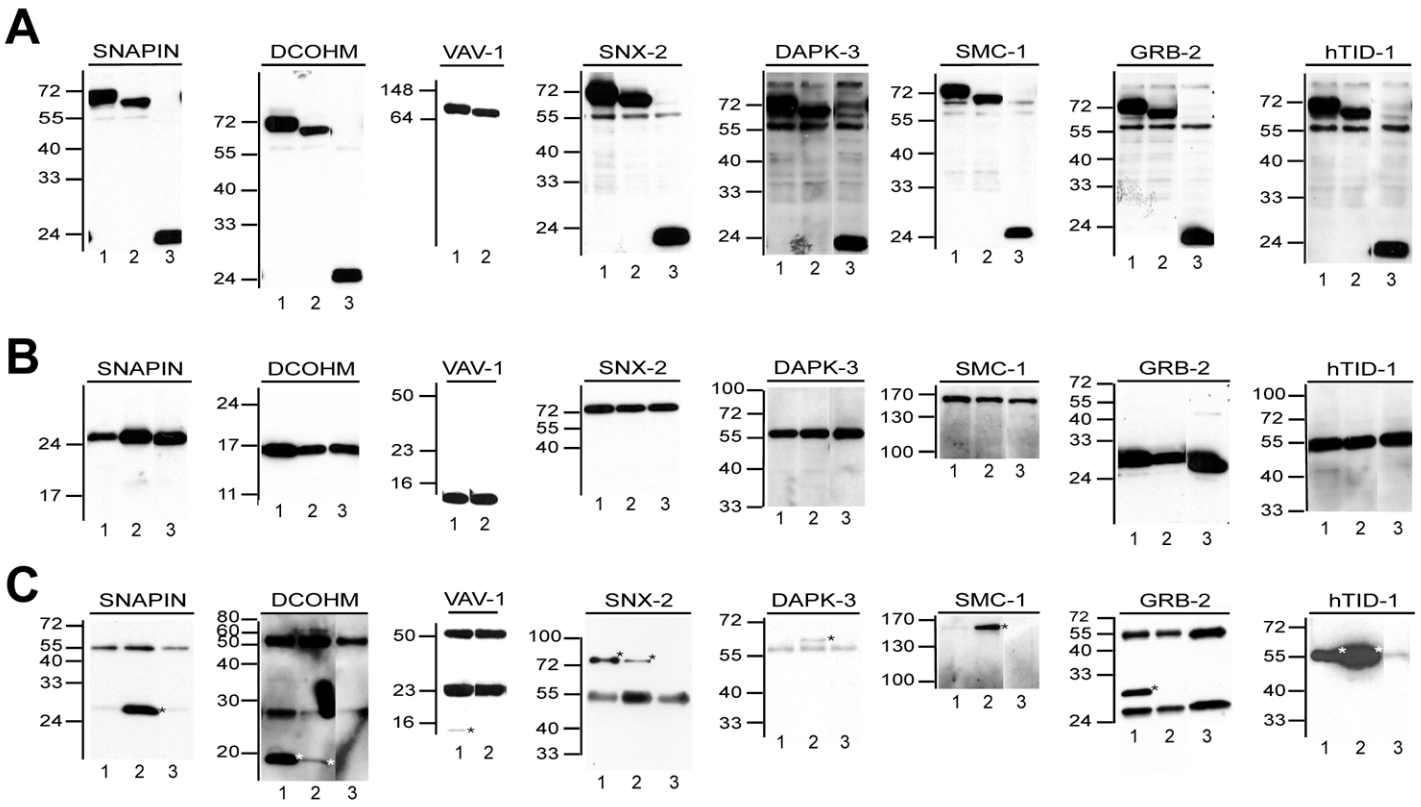

Figure 3. Coimmunoprecipitation of putative interaction partners with TPR/MET, the kinase negative TPR/MET K241A and TPR ΔMET. Total cell lysate was checked for proper expression of TPR/MET with anti-HA antibody (***A***) and for expression of the analyzed interaction partners with anti-Xpress, anti-myc (SNX2) or HRP-labeled anti-FLAG (SNAPIN) antibody (***B***). Corresponding to each interaction partner, column 1 shows cotransfection with pMT2SM-3xHA-TPR/MET; column 2 shows pMT2SM-3xHA-TPR/METK241A; and column 3 shows pMT2SM-3xHA-TPRΔMET. ***A***) The mutated TPR/MET protein as one single band with an approximate molecular weight of 64 kDa and the wild-type TPR/MET also as one single band with a slightly larger size because all TPR/MET proteins are phosphorylated on tyrosine. pMT2SM-3xHA-TPRΔMET codes for a small portion of the TPR protein (that part of the TPR protein present in the TPR/MET fusion protein) with an approximate molecular weight of 15 kDa. Coprecipitated proteins were detected using anti-Xpress (Invitrogen) or anti-myc antibodies and HRP-conjugated secondary antibodies (Bio-Rad). SNAPIN was directly detected using HRP-labeled anti-FLAG antibody (Sigma). Bands at 50 kDa and 23 kDa resemble the heavy and light chain of the precipitating antibody. Coimmunoprecipitated proteins are marked by a black or white asterisk. hTID-1 and SNAPIN run at the same position as the heavy and light chains of immunoglobulin, respectively. However, compared with the control (TPR alone), the signals of hTID-1 and SNAPIN in TPR/MET and/or TPR/MET K241A (***C***) are much stronger. For VAV-1 coimmunoprecipitation analysis, no pMT2SM-3xHA-TPRΔMET control is available. However, coimmunoprecipitation analysis of VAV-1 with pMT2SM-3xHA-TPR/METK241A shows no interaction, implying that VAV-1 does not interact with the inactive/kinase-negative receptor as well as with the TPR portion alone. The position of migration and sizes (in kDa) of the protein molecular weight markers are indicated in the left borders.