Receptor tyrosine kinases (RTKs) control many fundamental cellular processes, such as cell proliferation, differentiation, migration, and metabolism. RTK activity normally is under tight control, and dysregulated RTK activation is associated with most cancers, making RTKs important targets for cancer therapy (1). RTKs allow the cell to respond to external cues; ligand binding to the extracellular domain (ECD) of RTKs results in transphosphorylation of their cytoplasmic domains, which in turn leads to downstream signaling. The prevailing model of RTK activation states that receptors are monomeric in the absence of ligand but become dimerized upon ligand binding; dimerization brings the cytoplasmic domains in close proximity, favoring transphosphorylation (2). However, some studies have found dimerized RTKs in the absence of ligand, suggesting that activation may involve ligand-induced conformational changes within a dimeric receptor (e.g. Refs. 3–5).

The discoidin domain receptor (DDR) family of RTKs consists of two members, DDR1 and DDR2, that are characterized by the presence of an extracellular discoidin homology (DS) domain. Uniquely among RTKs, the DDRs are activated by a major extracellular matrix component, triple-helical collagen (6, 7). Several collagen types bind to and activate the DDRs, with the two receptors displaying different specificities toward certain collagen types (6–8). The DDRs are widely expressed in normal and malignant tissues and control developmental processes; DDR1 is essential for mammary gland development in the mouse (9), and DDR2 controls bone growth through chondrocyte proliferation (10). Both receptors regulate cell proliferation, adhesion, and motility and control remodeling of the extracellular matrix by regulating the expression and activity of matrix metalloproteinases (11–14). With respect to human disease, the DDRs are associated with cancer (15–20), fibrotic diseases of the lung and liver (12, 21), atherosclerosis (14), and osteoarthritis (22).

DDR1 and DDR2 share the same domain architecture: an ECD consisting of an N-terminal DS domain followed by a unique sequence of ~200 amino acids; a single-span transmembrane (TM) domain; an unusually large cytosolic juxtamembrane domain; and a C-terminal tyrosine kinase domain. In a previous study, we found that DDR activation, manifested by receptor autophosphorylation, is a consequence of collagen binding to a specific site within the DDR DS domain (23). We also observed that collagen binding by the DDRs requires dimerization of their ECDs (23), but how this observation relates to the oligomeric state of full-length DDRs within the cell membrane and the activation mechanism remained unclear.

In the present study, we have analyzed the oligomerization state of full-length DDRs in their natural environment, the
mammalian cell membrane. We show that the DDRs form ligand-independent dimers, both in the endoplasmic reticulum and at the cell surface. Neither the ECD nor the cytoplasmic domain of DDR1 is required for this interaction. In contrast, a leucine zipper motif in the DDR1 TM domain mediates strong self-association in a bacterial cell membrane and is essential for DDR1 activation in mammalian cells. Our findings demonstrate that the activation mechanism of DDRs is unlikely to involve ligand-induced receptor dimerization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human embryonic kidney (HEK) 293 cells were cultured as described (23).

**Chemicals and Reagents**—Rat tail collagen I (C-7661) was from Sigma (Poole, UK). Bis(sulfsuccinimidyl) suberate (BS6) and sulfo-NHS-LC-biotin were from Pierce. Protein A and protein G-Sepharose beads were from Amersham Biosciences (UK; Chalfont St. Giles, UK). The antibodies and their sources were as follows: rabbit anti-DDR1 (sc-532), goat anti-DDR2 (sc-7554), and rabbit anti-Myc from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); mouse anti-FLAG, M2, from Sigma; rabbit anti-maltose-binding protein (MBP) from New England Biolabs (Hitchin UK); anti-phosphotyrosine, clone 4G10, from Upstate Biotechnology (Lake Placid, NY). The anti-DDR1 Ab 74A, against the ECD, was a kind gift from Dr. Michel Faure, SUGEN Inc, San Francisco, CA. Secondary Abs and sulfo-NHS-LC-biotin were from Pierce. Protein A and Protein G-Sepharose beads were from Amersham Biosciences UK (Chalfont St. Giles, UK). The antibodies and their sources were as follows: rabbit anti-DDR1 (sc-532), goat anti-DDR2 (sc-7554), and rabbit anti-Myc from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); mouse anti-FLAG, M2, from Sigma; rabbit anti-maltose-binding protein (MBP) from New England Biolabs (Hitchin UK); anti-phosphotyrosine, clone 4G10, from Upstate Biotechnology (Lake Placid, NY). The anti-DDR1 Ab 74A, against the ECD, was a kind gift from Dr. Michel Faure, SUGEN Inc, San Francisco, CA. Secondary Abs were as follows: sheep anti-mouse IgG-horseradish peroxidase (Amersham Biosciences); goat anti-rabbit IgG-horseradish peroxidase (Dako, Ely, UK); rabbit anti-goat IgG-horseradish peroxidase (Zymed Laboratories Inc., San Francisco, CA).

**DNA Constructs**—Restriction and modification enzymes were purchased from New England Biolabs or Promega (Southampton, UK). All PCR amplification reactions were performed with Pfu DNA polymerase (Stratagene, Amsterdam, The Netherlands). All PCR-derived sequences were verified by DNA sequencing. All constructs for expression in HEK293 cells were subcloned into the mammalian expression vector pcDNA3.1/zeo (Invitrogen). PCR primers used for generating the mutant constructs can be obtained on request. The generation of the DDR1 deletion constructs DS1-1, DS1-2, and ΔDS1 was described previously (23). The same method was used for the generation of the ΔGG construct, eliminating residues Asn-370–Ala-411. The cytoplasmic DDR1 deletion construct MDN (DDR1b truncated after Arg-525) was a gift from Dr. Michel Faure, SUGEN Inc. The cytoplasmic deletion construct ECTM was made by PCR amplification from full-length DDR1b, introducing a STOP codon after Arg-445. The same PCR primers were used to generate the two constructs DS1-2ΔCYT and ΔDS1ΔCYT, but amplification was from the DS1-2 and ΔDS1 constructs, respectively. The TM mutations were constructed by overlap extension PCR using mutagenic primers that introduced the desired point mutations.

The TOXCAT constructs were made as follows. The expression vectors pccKAN, pccGpa-wt, and pccGpa-831 and the *Escherichia coli malE* strain MM39 (24) were a kind gift of Dr. Donald Engelman (Yale University, New Haven, CT). The genes coding for the chimeric proteins with DDR1 TM domains were generated by annealing of custom-designed, complementary oligonucleotides, which were subsequently cloned in-frame into a Nhel-BamHI digested pccKAN vector. The resulting ToxR′ (DDR1-TM)MBP constructs were verified by DNA sequencing and transformed into MM39 cells.

**Chemical Cross-linking of Surface-expressed Receptors**—Cross-linking was carried out for 30 min at room temperature using the homobifunctional reagent BS3. Different concentrations of BS3 in a final volume of 500 μl of PBS were added to confluent cells in 12-well tissue culture plates. The reaction was quenched by the addition of 20 mM Tris, pH 7.4, and incubating at room temperature for 15 min followed by cell lysis.

**Co-immunoprecipitation**—HEK293 cells at 50% confluency were transfected by calcium phosphate precipitation, as described (23). Cells were co-transfected with plasmid vectors containing DDR-Myc and DDR-FLAG. Single transfections, with either one of the plasmids alone, were also carried out as controls. 48 h later, cells were lysed on ice in 1% Nonidet P40, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin. The detergent soluble fraction was recovered by centrifugation at 15,000 × g, and supernatants were subjected to immunoprecipitation with either rabbit anti-Myc or mouse anti-FLAG Abs. Immune complexes were isolated with protein A or protein G conjugated to Sepharose beads, respectively. The immunoprecipitates were washed four times with lysis buffer. The proteins were analyzed by SDS-PAGE on 7.5% polyacrylamide gels followed by blotting onto nitrocellulose membranes. The blots were probed with either rabbit anti-Myc or mouse anti-FLAG followed by relevant secondary Abs conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence (Amersham Biosciences).

**Cell Surface Biotinylation**—Cell surface biotinylation of HEK293 cells expressing DDR1 constructs was carried out as follows. The cells were incubated with sulfo-NHS-LC-biotin in PBS for 30 min on ice. The reaction was quenched by washing twice with 100 mM glycine in cold PBS. The cells were lysed in Nonidet P-40 lysis buffer (as above), and one aliquot of cell lysate was incubated for 2 h with streptavidin-Sepharose beads to bind biotinylated cell surface receptors. The beads were pelleted by centrifugation, and the recovered supernatant was incubated for another 2 h with fresh streptavidin-Sepharose beads to remove any remaining biotinylated cell surface receptors. The beads from the first and second incubation were pooled and washed four times in Nonidet P-40 lysis buffer and then boiled in reducing SDS sample buffer to release bound protein. The supernatant from the second incubation was recovered and incubated with anti-DDR1 Ab to immunoprecipitate the intracellular DDR receptor pool. To analyze the total DDR protein in the sample, another aliquot of starting lysate of equal volume was incubated with anti-DDR1 Ab for DDR immunoprecipitation. As a control for an intracellular protein, ERK2 was chosen. Samples were separated by 7.5% SDS-PAGE and analyzed by Western blotting with anti-DDR1 or anti-ERK2 Ab.

The generation of the DDR1 deletion constructs DS1-1, DS1-2, and ΔDS1 was described previously (23). The same method was used for the generation of the ΔGG construct, eliminating residues Asn-370–Ala-411. The cytoplasmic DDR1 deletion construct MDN (DDR1b truncated after Arg-525) was a gift from Dr. Michel Faure, SUGEN Inc. The cytoplasmic deletion construct ECTM was made by PCR amplification from full-length DDR1b, introducing a STOP codon after Arg-445. The same PCR primers were used to generate the two constructs DS1-2ΔCYT and ΔDS1ΔCYT, but amplification was from the DS1-2 and ΔDS1 constructs, respectively. The TM mutations were constructed by overlap extension PCR using mutagenic primers that introduced the desired point mutations.

The TOXCAT constructs were made as follows. The expression vectors pccKAN, pccGpa-wt, and pccGpa-831 and the *Escherichia coli malE* strain MM39 (24) were a kind gift of Dr. Donald Engelman (Yale University, New Haven, CT). The genes coding for the chimeric proteins with DDR1 TM domains were generated by annealing of custom-designed, complementary oligonucleotides, which were subsequently cloned in-frame into a Nhel-BamHI digested pccKAN vector. The resulting ToxR′ (DDR1-TM)MBP constructs were verified by DNA sequencing and transformed into MM39 cells.

**Collagen-dependent DDR Autophosphorylation**—This assay was performed as described (23). Briefly, HEK293 cells in 12-well plates were transfected by calcium phosphate precipi-
tation with the relevant DDR expression vectors. 24 h later, the cells were incubated with serum-free medium for 16 h. Cells were then stimulated with 10 μg/ml collagen for 90 min at 37 °C. Cells were lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF. Aliquots of the lysates were analyzed by SDS-PAGE followed by blotting onto nitrocellulose membranes. The blots were probed with mouse anti-phosphotyrosine mAbs followed by sheep anti-mouse Ig horseradish peroxidase. Detection was by enhanced chemiluminescence (Amersham Biosciences). To strip the blots, membranes were incubated in either 62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol at 55 °C for 30 min or antibody stripping solution (Alpha Diagnostic International, San Antonio, TX) for 10 min at room temperature. The blots were reprobed with rabbit anti-DDR1 or goat anti-DDR2 Abs followed by goat anti-rabbit Ig-horseradish peroxidase or rabbit anti-goat Ig-horseradish peroxidase.

Chloramphenicol Acetyltransferase (CAT) ELISA—To generate cell-free extracts for the CAT ELISA, single colonies of transformed MM39 cells were inoculated into 5 ml of M9 minimal medium containing 0.4% maltose as a unique source of carbon and 100 μg/ml ampicillin. The bacterial cultures were grown at 37 °C with vigorous shaking to A600 of 0.6–0.7 and then harvested by pelleting 1 ml of culture at 250 × g for 10 min at 4 °C. The cell pellets were washed twice with PBS at 4 °C and finally resuspended in 1 ml of lysis buffer (Roche Applied Science). The cells were lysed for 30 min at room temperature, and 75 μl of the resulting cell-free extracts were assayed for CAT concentration using a CAT ELISA kit (Roche Applied Science) according to the manufacturer’s instructions.

Expression of TOXCAT Proteins—Single colonies of transformed MM39 cells were grown in LB broth with 100 μg/ml ampicillin to A600 of ~0.6. Cells were pelleted from 0.5 ml of bacterial culture and resuspended in PBS. Cells were lysed by the addition of SDS sample buffer and boiling for 5 min. The samples were separated by 10% SDS-PAGE, blotted, and probed with anti-MBP as primary Ab followed by anti-rabbit Abs conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

Ligand-independent DDR Dimerization—To investigate whether the DDRs exist as preformed oligomers in the absence of collagen, we performed chemical cross-linking experiments. HEK293 cells were transiently transfected with human DDR1 (DDR1b isoform) or DDR2 and incubated in the presence or absence of the membrane-impermeable, homobifunctional cross linker BS3. The DDRs were detected by Western blotting of cell lysates. In the absence of cross-linker, DDR1 is observed to migrate as two species of ~120 and 125 kDa (Fig. 1A). The lower molecular weight species represents the high mannose, biosynthetic precursor of the receptor, whereas the upper band represents complex glycosylated DDR1 (data not shown and Ref. 25). In the presence of BS3, an ~240-kDa band, corresponding to the size of a DDR1 dimer, was detected by the anti-DDR1 Ab. Likewise, for DDR2, which migrates as a mixture of three species, with the upper two forms representing complex glycosylated mature forms (data not shown), a band appeared in the presence of BS3, which corresponds to the size of dimeric DDR2. These results indicate that the DDRs, or at least a sizeable fraction of molecules, exist as dimers on the cell surface in the absence of ligand.

To confirm the formation of DDR homodimers, we performed co-immunoprecipitation studies, using two differently epitope-tagged DDR constructs. Both DDR1 and DDR2 were tagged at the C terminus with either a Myc tag or a FLAG tag. Upon transient transfection into HEK293 cells, these receptor constructs were expressed with the same efficiency as their wild-type counterparts, exhibited the same pattern of protein bands, and showed collagen-dependent autophosphorylation indistinguishable from the wild-type receptors (data not shown). Following transient expression in HEK293 cells, Myc- and FLAG-tagged DDR1 could be immunoprecipitated,
respectively, with anti-Myc (Fig. 1B, lane 4) and anti-FLAG Ab (data not shown). The immunoprecipitation was specific since anti-Myc and anti-FLAG Ab did not precipitate, respectively, DDR1-FLAG (Fig. 1B, lane 1) and DDR1-Myc (data not shown). For co-immunoprecipitation assays, DDR1-Myc and DDR1-FLAG were co-transfected into HEK293 cells. Cell lysates were immunoprecipitated with anti-Myc Ab, and the precipitated material was analyzed by Western blotting with anti-Myc or anti-FLAG Ab. Fig. 1B, lane 2, shows that DDR1-FLAG co-precipitated with the anti-Myc Ab, indicating that DDR1-Myc and DDR1-FLAG associate with one another. Importantly, co-precipitation only occurred in lysates from cells co-transfected with both types of tagged DDR1 but not when lysates of singly transfected cells were mixed before immunoprecipitation (Fig. 1B, lane 3, asterisk). This indicates that co-precipitation requires the tagged receptors to be expressed on the same cell membrane and rules out nonspecific receptor association after cell lysis. It is notable that both the precursor (high mannose) and the mature forms of DDR1-FLAG co-precipitate with DDR1-Myc expressed in the same cell (Fig. 1B, lane 2). Similar results were obtained with DDR2 (Fig. 1B). We obtained equivalent results when the protocol was reversed, and immunoprecipitation of cells co-expressing the two epitope-tagged forms of the DDRs was performed with the anti-FLAG Ab followed by Western blotting with the anti-Myc Ab (data not shown). From these experiments, we conclude that DDR1 and DDR2 associate to form homodimers and that dimerization already takes place during biosynthesis, preceding the appearance of DDRs on the cell surface.

We also investigated whether collagen binding increases the amount of DDR dimers on the cell surface. Cells co-expressing both types of tagged DDRs were subjected to co-immunoprecipitation analysis in the presence or absence of collagen stimulation. For both DDR1 and DDR2, there was no increase in the amount of DDR co-immunoprecipitation (Fig. 2 and data not shown), indicating that collagen binding does not increase DDR dimerization on the cell surface.

Neither the Extracellular nor the Cytoplasmic Domain of DDR1 Is Essential for DDR1 Dimerization—We next wished to determine which structural domains in DDR1 are responsible for homodimerization. We made a set of deletion constructs and subjected them to dimerization analysis by both cross-linking and co-immunoprecipitation. The extracellular domain of DDR1 is composed of an N-terminal DS domain followed by a group of acidic residues in DDR1b) followed by the tyrosine kinase domain. Notably, neither the cytoplasmic domain nor individual subdomains of the DDR1 ECD were found to be essential for dimerization as none of the deletion constructs was defective in the co-immunoprecipitation assay (Fig. 3A and data not shown). The cytoplasmic deletion constructs were readily cross-linked by BS3, with prominent bands corresponding to the expected molecular weights of the shortened proteins (Fig. 3B and Supplemental Fig. 1; see the legend for Supplemental Fig. 1 for molecular size determination). The appearance of cross-linked bands corresponding to the expected dimer sizes of these truncated proteins provides strong evidence that the cross-linking assay detects DDR homodimers rather than hetero-oligomers with another unknown protein. Not all of the extracellular deletion mutants could be cross-linked (Fig. 3A and data not shown). We therefore used cell surface biotinylation to establish which of the deletion mutants were localized to the cell surface. There was a strict correlation between the ability of the truncated DDR1 proteins to be cross-linked by BS3 and their surface localization (Fig. 3, A and E), showing that the cross-linking assay indeed detects only surface-localized DDR1. To rule out that DDR1 dimerization is due to multiple interactions along the entire ECD (in which case individual domain deletions within a homodimer might be tolerated), we co-expressed two different ECD deletion mutants that are not expected to be able to interact via their ECD. The DS1-2 construct retains the DDR1 DS domain but places it in an unnatural position close to the TM domain. The co-expressed ΔDS1 construct, on the other hand, is missing the DS domain and retains the rest of the DDR1 ECD. Co-immunoprecipitation studies (Fig. 3C) indicated no impairment in the ability of these proteins to associate, showing that the entire DDR1 ECD is dispensable for DDR1 dimerization. A construct bearing only the DS domain in the ECD with the entire cytoplasmic domain deleted also showed homodimeric interaction in the co-immunoprecipitation assay (Fig. 3D), indicating that, in the absence of the cytoplasmic domain, the DS domain and the TM domain are sufficient for DDR1 dimerization.
A Transmembrane Leucine Zipper Required for DDR1 Function

The Role of the DDR1 TM Domain in Dimerization and Signaling—As the DDR1 TM domain is common to all of the deletion constructs tested in our dimerization assays, it seemed likely that the TM domain contributes to interactions between the DDR1 monomers. The DDR1 TM domain contains two putative dimerization motifs (Fig. 4A). One is a GXXXG motif (GXXXA in the DDRs) (26, 27), which is the principal component of the dimerization interface of the strongly dimerizing TM domain of glycophorin A (GpA) (28). GXXXG motifs are also important for TM interactions of ErbB receptors (29). In addition to the GXXXG motif, the DDR1 TM sequence also contains heptad motifs of (iso)leucine residues, which are thought to mediate TM domain dimerization in a similar manner as in leucine zippers (30, 31). A dimeric leucine zipper is a parallel coiled-coil composed of two α-helices interacting through apolar residues in positions a and d of a 7-residue helical repeat (32).

To test which (if any) of the potential dimerization motifs are important for DDR1 dimerization or TM signaling, we created three DDR1 TM mutants (Fig. 4A). To disrupt association via the leucine zipper, we introduced Gly-Pro residues (mutants TM1 and TM2), similarly to previous work on E-cadherin (33) and the erythropoietin receptor (34). The 5-residue GXXXG motif tolerates only the smallest amino acids (Gly or Ala) in the position of the 2nd Gly (26). To disrupt association via the GXXXG motif, (GXXXA in DDR1) the Ala residue corresponding to the 2nd Gly was mutated to Val (mutant TM3), similarly to previous work on ErbB receptors (29). Importantly, the TM3 mutation is compatible with formation of a leucine zipper. The mutant constructs were made in the context of full-length DDR1 (untagged or Myc- and FLAG-tagged). We first tested the mutant constructs for their ability to dimerize. All of the mutants showed dimerization to the same extent as wild-type DDR1, as measured both by cross-linking of cell surface receptors with BS5 (Fig. 4B) and by co-immunoprecipitation of FLAG- and Myc-tagged receptors (Fig. 4C). Thus, neither the GXXXG motif nor the leucine zipper motif is essential for dimerization of full-length DDR1. Fig. 4C shows co-immunoprecipitation of an ~60-kDa DDR1-reactive band that corresponds to truncated DDR1 lacking almost the entire ECD (35). This fragment was also observed with wild-type DDR1 (data not shown), and its presence in the co-immunoprecipitate further demonstrates that the ECD is not required for DDR1 dimerization.

Collagen-induced activation of DDR1 can be followed by monitoring receptor autophosphorylation (6, 7). The effect of the TM mutations on collagen-induced DDR1 phosphorylation was analyzed in transiently transfected HEK293 cells, which were incubated with different concentrations of collagen I. The leucine zipper mutations, TM1 and TM2, strongly impaired receptor activation at all collagen concentrations tested (Fig. 5A). In contrast, the GXXXG mutant, TM3, showed robust autophosphorylation, similar to wild-type DDR1 (Fig. 5B). These results demonstrate that the leucine zipper motif in the DDR1 TM domain, but not the GXXXG motif, is essential for DDR1 signaling, suggesting that during TM signaling, the DDR1 TM domains are associated via a membrane-spanning leucine zipper.

The DDR1 Transmembrane Domain Self-associates in Biological Membranes—To establish whether the DDR1 TM domain can indeed self-associate via a membrane-spanning leucine zipper in a biological membrane, we used the TOXCAT

FIGURE 3. Neither the cytoplasmic domain nor the ECD of DDR1 is essential for ligand-independent dimerization. A, schematic representation of DDR1 deletion constructs. All constructs were transiently expressed in HEK293 cells and assessed for their ability to homodimerize by co-immunoprecipitation of Myc- and FLAG-tagged receptors and cross-linking of surface-localized, untagged receptors with BS5. All constructs were expressed by the cells except ΔDS1 ΔCYT, which could not be detected with relevant Abs. A summary of the results is given. P/G rich, Pro/Gly-rich; ColP, co-immunoprecipitation assay; XL, cross-linking assay; Surface, cell-surface localization using a biotinylation assay; ND, not determined. B, cross-linking of cytoplasmic deletion mutants MDN and ECTM, carried out as in Fig. 1A. Cell lysates were analyzed by Western blotting using an anti-DDR1 Ab against the ECD. The arrowhead points to the position of cross-linked MDN dimer. See supplemental Fig. 1 for estimation of molecular weights. mock, mock-transfected. C, co-immunoprecipitation assay of ΔDS1-Myc with DS1-2-FLAG, carried out as in Fig. 18. D, co-immunoprecipitation assay of DS1-2-CYT-Myc with DS1-2-CYT-FLAG, carried out as in Fig. 18, except that cell lysates were immunoprecipitated (IP) with anti-FLAG Ab. The arrowhead indicates the DS1-2-CYT protein, which is close in size to the Ig light chain. E, cell surface biotinylation of HEK293 cells expressing DDR1 constructs, showing surface localization of wild-type protein but not of its ΔPG deletion mutant. Cell surface proteins were biotinylated and precipitated with streptavidin-Sepharose beads from cell lysates. The supernatant from the pull-down was recovered and incubated with anti-DDR1 Ab to immunoprecipitate the intracellular DDR1 receptor pool. ERK2 served as a control for an intracellular protein. Samples were separated by 7.5% SDS-PAGE and analyzed by Western blotting with anti-DDR1 or anti-ERK2 Ab as indicated. T, total DDR1 or ERK2 in the lysates; S, surface pool (material released from streptavidin beads); I, intracellular pool (immunoprecipitated from supernatant after streptavidin bead incubation). The experiments were carried out three times (B and D) or twice (C and E) with very similar results.
A transmembrane leucine zipper required for DDR1 function.

The TM sequence of DDR1 is required for dimerization.

FIGURE 4. Mutations of the putative dimerization motifs in the DDR1 TM domain do not impair ligand-independent DDR1 dimerization. A, amino acid sequences of wild-type (wt) and mutant DDR1 TM domains. The heptad repeat pattern of (iso)leucine residues (see “Results”) is indicated above the sequence. The region corresponding to the potential dimerization motif GXXXG is shaded gray, B, cross-linking of DDR1 wild-type and TM domain mutants, TM1–3, carried out as in Fig. 1A. C, co-immunoprecipitation assays of DDR1 TM mutants, TM1 and TM3, carried out as in Fig. 18. The arrowheads point to the position of cleaved DDR1, which consists of the TM and cytoplasmic domain with most of the ECD deleted. The experiments were carried out a minimum of three times with very similar results.

system developed by Russ and Engelmann (24). In this system, a chimeric protein consisting of an N-terminal ToxR’ DNA-binding protein, the TM domain of interest, and a C-terminal MBP domain is expressed in the inner membrane of E. coli. Dimerization via the TM sequence drives cytoplasmic ToxR’ dimerization, resulting in transcriptional activation of a CAT reporter gene. CAT enzyme activity is measured and assumed to be proportional to the extent of TM helix-mediated dimerization.

In the TOXCAT assay, the length of the inserted TM sequence and the locations of the junctions between the TM helix and the ToxR’ and MBP are important determinants for the degree of CAT expression as the positioning of the TM helix in the membrane influences the orientation of the fused domains with respect to the TM dimer interface. We therefore first examined the effect of inserting different DDR1 TM sequences into the ToxR’ chimeric protein by sequentially deleting single amino acids from the TM domain (Fig. 6A). All tested DDR1 chimeric proteins showed strong induction of CAT activity (Fig. 6B). This CAT activity was similar to, or greater than, that of a control construct containing a 15-amino-acid GpA wild-type sequence, demonstrating a strong self-association of the DDR1 TM domain. Since CAT activity is also proportional to the amount of fusion protein expressed, we compared the expression levels of chimeric proteins by Western blotting of bacterial lysates (Fig. 6B, lower panel). All tested fusion proteins were expressed at similar levels. It is noticeable that although the GpA constructs showed some proteolyzed chimera (Fig. 6B, arrowhead, as observed in the original TOXCAT study (24)), no equivalent bands were observed for the DDR1 chimera. Thus, the DDR1 chimera appears to be more stable than the GpA chimera, possibly explaining the strong induction of CAT activity of the former compared with the latter.

To examine the effects of the DDR1 mutations TM1–3, described above, in the TOXCAT assay, we introduced the same mutations into the DDR1.1 ToxR’ fusion protein (chosen because it consistently generated the highest CAT activity in the TOXCAT assay). Fig. 6C shows that the two leucine zipper mutants, TM1 and TM2, exhibited strongly reduced CAT activity, whereas the GXXG mutant, TM3, had the same CAT activity as wild-type DDR1. The deleterious effect of the TM1 and TM2 mutants is most likely underestimated in the CAT activity assay as we consistently observed the TM1 and TM2 mutant chimeras to be expressed at higher levels than the wild-type and TM3 constructs (Fig. 6C, lower panel). These results demonstrate that the DDR1 TM helices self-associate in a bacterial cell membrane via a membrane-spanning leucine zipper.

DISCUSSION

A widely accepted mechanism of RTK activation invokes ligand-dependent receptor dimerization (2). Here we present evidence that the DDRs, an unusual family of RTKs activated by collagen, may respond to ligand binding by a conformational change within a preformed dimer that results in autophosphorylation of the cytosolic domains. We find that DDR dimerization already occurs during biosynthetic transport to the cell surface. We propose that preformed DDR dimers are the predominant species on the cell surface, consistent with our previous finding that dimerization of the DDR ECD is a prerequisite for collagen binding (23). Ligand-independent RTK dimerization is not without precedent. Several studies have detected preformed epidermal growth factor receptor dimers, suggesting that activation may result from conformational rearrangements within a dimer (3–5, 36). Moreover, insulin receptor is a covalent disulfide-linked dimer that is activated by ligand without further oligomerization (37). In cytokine receptors, which are believed to share many mechanistic features with RTKs, the ligand-induced dimerization model has recently been challenged by the findings that both the erythropoietin and the growth hormone receptors exhibit ligand-independent dimerization (38–40).

Which DDR1 subdomains are essential for dimer formation? Because we found that neither the ECD nor the cytoplasmic domain is required for dimerization, we focused on the TM domain, which contains two potential dimerization motifs, a GXXXG and a leucine zipper motif. The GXXG motif is frequently found in the TM domains of other RTKs (26) and has
been shown to promote TM helix dimerization of ErbB receptors (29, 41). Leucine zippers have been found to be important for functional oligomeric assembly of a number of single-span TM proteins, including the erythropoietin receptor (34, 42) and E-cadherin (33). Somewhat surprisingly, we found that disruption of neither motif in full-length DDR1 affected dimer formation. The collective results thus indicate that no single domain in DDR1 is solely responsible for dimerization but that multiple interactions along the entire DDR1 molecule (ECD, TM, and cytosolic domain) collaborate in dimer formation. Because it is difficult to study the isolated DDR1 TM domain in mammalian cells, we used the bacterial TOXCAT reporter assay to obtain a semiquantitative measure of TM dimerization (24). The DDR1 TM domain showed a strong propensity for dimerization, which was impaired by mutation of the leucine zipper motif but not of the GXXXG motif. Thus, the DDR1 TM domain self-associates in a biological membrane via the leucine zipper motif.

Although mutation of the TM leucine zipper failed to disrupt dimerization of full-length DDR1, it had a dramatic effect on DDR1 function. Mutation of the leucine zipper motif, but not of the GXXXG motif, reduced collagen-induced autophosphorylation of DDR1 to background levels, suggesting that specific interactions made by the leucine zipper are involved in transmitting the conformational change resulting from ligand engagement to the cytosolic kinase domains. Although we do not know the nature of this conformational change, a possible mechanism could involve the relative rotation of receptor protomers within the dimer, as suggested for the epidermal growth factor receptor (3), the erythropoietin receptor (43), and the growth hormone receptor (40). Another intriguing property of the DDRs still awaiting a mechanistic explanation is their unusually slow and sustained activation kinetics (6, 7). It seems unlikely that conformational changes within a DDR dimer occur on a time scale of minutes, and other, slower processes, such as receptor clustering, may be involved in DDR activation. In summary, our findings show that the DDRs exist as stable dimers in the absence of ligand and that receptor activation requires specific interactions made by the transmembrane leucine zipper.

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FIGURE 5. The DDR1 TM domain leucine zipper motif is necessary for TM signaling. A and B, collagen-induced autophosphorylation of DDR1 wild-type (wt) and TM mutants. Full-length wild-type DDR1 and its TM mutants, TM1–3, were transiently expressed in HEK293 cells. After stimulation with collagen I (Coll), cell lysates were analyzed by SDS-PAGE and Western blotting. The blots were probed with anti-phosphotyrosine (anti-PY) mAb 4G10 (upper panels), stripped, and reprobed with an Ab against the C terminus of DDR1 (lower panels). Collagen I was used at different concentrations, as indicated (in μg/ml). The experiment was carried out three times with very similar results. mock, mock-transfected.

FIGURE 6. The DDR1 TM domain self-associates in bacterial membranes. A, DDR1 TM sequences tested in the TOXCAT assay (24). The dimerizing GpA sequence and the non-dimerizing G83I GpA mutant were used as positive and negative controls, respectively. wt, wild type. B, homodimerization of ToxR’ chimeric constructs, as measured by CAT enzyme activity. Lysates from MM39 E. coli cells expressing chimeras with the indicated TM sequences were used to measure CAT activity by ELISA. CAT activity is represented as absorbance at 405 nm. The experiment is representative of three independent experiments. The lower panel shows the relative expression levels of the chimeric proteins. Lysates from MM39 cells expressing the chimeric constructs were separated on 10% SDS-PAGE and immunoblotted with an Ab against the C-terminal MBP domain. The asterisk denotes full-length chimeras; the arrowhead denotes proteolyzed GpA chimeras. C, homodimerization of ToxR’ chimeric constructs containing wild-type and mutant DDR1 TM sequences, determined as in B. The data are presented as mean ± standard deviation from three independent experiments, each performed in triplicate or quintuplicate and normalized to the values obtained with DDR1.1. The lower panel shows the relative expression levels, as in B; UT, untransformed cells.
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