Enhancement of the Antagonistic Potency of Transforming Growth Factor-β Receptor Extracellular Domains by Coiled Coil-induced Homo- and Heterodimerization*

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Transforming growth factor-β (TGF-β) plays a causal role in several human pathologies including fibrotic diseases and metastasis. TGF-β signaling is mediated through its interaction with three types of cell surface receptors, RI, RII, and RIII. The soluble ectodomains of RII and RIII bind to TGF-β, making them attractive candidates to sequester TGF-β and inhibit its activity. To optimize the activity of the ectodomains, we studied the effect of artificially dimerizing them upon their kinetics of binding to TGF-β using an optical biosensor and studied their antagonistic potencies using an in vitro signaling assay. We fused the RII ectodomain and the membrane-proximal ligand-binding domain of the RIII ectodomain to de novo designed heterodimerizing coil strands and demonstrated that the coil strands within the fusion proteins were capable of promoting the dimerization of the coil-tagged ectodomains. Our results indicate that coiled-coil-induced dimerization of the ectodomains stabilized their interaction with TGF-β as compared with the monomeric ectodomains. Also, in contrast to the monomeric ectodomains, which did not block signaling, the coiled-coil-induced dimers were characterized by antagonistic potencies in the low nanomolar range.

Mammalian transforming growth factor-β isoforms (TGF-β1, -β2, and -β3) mediate signaling by binding to and complexing three types of cell surface receptors known as the TGF-β type I (RI), type II (RII), and type III (RIII) receptors (1). In the absence of ligand, both RI and RII can form homooligomers (2). TGF-β1 and TGF-β3 bind simultaneously to two RII ectodomains (RIIEDs) (3). This binding event is thought to reorient the RIIs at the cell surface, allowing for the formation of a signaling-competent RI-RII complex (4). RIII was first thought to be an “accessory” receptor whose role is to present TGF-β to RII and RIII (5). Recent studies suggest, however, that the role of RIII is more complex. Firstly, it was demonstrated that RIII is required for TGF-β1-promoted mesenchymal transformation during chick embryonic heart development (6). Furthermore, it was shown that the RIII cytoplasmic domain is important for signaling (7) and that the role of RIII in signaling may be modulated by its glycosylation state (8). Two independent TGF-β-binding domains have been identified within the RIII ectodomain (9–11), further emphasizing the complexity of this receptor.

TGF-β is involved in the regulation of many physiological processes including cell growth and differentiation. TGF-β overexpression plays a key role in several human pathologies including fibrotic diseases (12) and metastasis (13). Recently, two reports provided encouraging results showing that a TGF-β antagonist, corresponding to the RIIIED artificially dimerized through the Fc portion of an antibody (RIIED-Fc), reduces metastasis in vivo without any adverse side effects (14, 15). Other soluble TGF-β-binding proteins, including the ectodomain of RIII, have also been evaluated for their antagonistic potency (16–19). We reported that their in vitro antagonistic potency inversely correlates with their rate of dissociation from TGF-β (16). The best antagonist found so far is RIIIED-Fc, which blocks TGF-β1 and -β3 signaling in vitro with an IC_{50} in the picomolar range (16, 18). These findings raise the interesting question of whether the antagonistic potency of the extracellular domains of other TGF-β receptors can be increased by artificially dimerizing them, as in the case of RIIIED-Fc.

In this report, we use a de novo designed heterodimerizing coiled-coil system (the E5/K5 coiled coil) that we have characterized (21) in order to rapidly produce and evaluate the effect of receptor ectodomain homo- and heterodimerization on their ability to bind to TGF-β and to antagonize signaling. This coiled-coil system is an ideal dimerization domain since the coils are small (35 amino acids) and specifically heterodimerize, with the resulting non-covalent coiled-coil complex being stable. We have previously produced and characterized a chimera composed of RIIIED fused to the E5 coil (RIIED-E5), which retained the same kinetics of binding to TGF-β as untagged RIIIED (22). In the present study, we report the expression of two new fusion proteins containing the K5 coil, i.e. RIIIED-K5 and the fusion of the K5 coil to the membrane-proximal ligand-binding domain of RIIIED (MP-RIIED-K5). We investigated the binding of these fusion proteins to TGF-β1 using a surface plasmon resonance (SPR)-based biosensor. We showed that the coil domains were able to promote the dimerization of RIIIED-E5 with MP-RIIED-K5 or with RIIIED-K5. We also analyzed the effect of coiled-coil-induced RIIIED and
MP-RIIED/RIIED dimerization both on binding to TGF-β1 and on the potency of the ectodomains to block TGF-β1 signaling in vitro. Our results indicate that the dimers have better antagonistic potencies than the corresponding monomers.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pcDNA3 vectors containing the cDNA encoding for the K5 coil (pcDNA3-K5coil) and for the N-terminally myc-tagged RII receptor (pcDNA3-RIII) were a generous gift from M. Banville and M. Jaramillo (Biotechnology Research Institute, Montreal, Quebec, Canada). The pcDNA3 vector containing the myc-tagged MP-RIIED (pcDNA3-Δ44–576) is described by Pepin et al. (23). All the enzymes were from New England Biolabs Inc. All the primers were purchased from Hokabell Scientific Ltd. (Montreal, Quebec, Canada). Recombinant human TGF-β1 and the anti-hRII antibody were from R&D Systems Inc. (Minneapolis, MN). Recombinant RIIED, expressed in *Escherichia coli*, purified, and refolded (3), was a generous gift from Dr. A. Hinck. The expression vector pTT2 has been described previously (22). The BIACORE 3000, CMS sensor chips, and reagents for amine coupling were from BIACORE Inc. (Piscataway, NJ).

**Construction of the RIIED-K5 and MP-RIIED-K5 Expression Vectors**—pTT2-R5 coil construction was performed as described by De Crescenzo et al. (22) using the pcDNA3-R5 coil as template; K forward, 5′-TAGACCGGCGGCCGCTAAGCTATTACGAGG-3′, and K reverse, 5′-TAACAGCGGCGGCCGCTAAGCTATTACGAGG-3′, as primers; and NotI and BamHI enzymes for digestion. For construction of pTT2 RIIED-K5, the cDNA encoding for the myc-tagged RIIED was PCR-amplified, digested, and ligated to pTT2-R5 coil as described previously (22). For construction of pTT2 MP-RIIED-K5, the cDNA encoding for the myc-tagged MP-RIIED was PCR-amplified using the pcDNA3-Δ44–575 as template and using the following primers: III forward, 5′-ATGCTAACGCTTGGAGATGCACGACATCC-3′, and III reverse, 5′-TAGACGCGGCACCATGGAAATCTCGTGTAGG-3′. The resulting fragment was digested with Nhel/NotI and ligated to pTT2-R5 coil digested with the same enzymes. pTT2 RIIED-K5 and pTT2 MP-RIIED-K5 transformation and purification were performed as described previously (22).

**Protein Production, Purification, and Concentration Determination**—Protein production, purification, and concentration determination were performed as described previously (22). The yields of RIIED-K5 and MP-RIIED-K5 from 500 ml of conditioned media were ~570 and 600 μg, respectively. The purity of the fusion proteins was estimated by silver staining using the Silver Stain Plus kit (Bio-Rad) after resolving the proteins on 11% SDS-polyacrylamide gels under reducing conditions. The purified proteins were also detected by Western blot (anti-myc 9E10, Santa Cruz Biotechnology, Inc.) following protein separation on 11% SDS-polyacrylamide gels under reducing and non-reducing conditions.

Higher order aggregates were observed by Western blot under non-reducing conditions for RIIED-K5. Monomeric RIIED-K5 was prepared as follows. RIIED-K5 (320 μg) was diluted in phosphate-buffered saline to a final volume of 10 ml and spun in a Centriprep 30 (Amicon). The filtrate was then concentrated using a Centriprep 10, leading to a 500-μl fraction with a RIIED-K5 concentration of 555 μg/ml. The efficacy of the separation of oligomers from monomer was estimated by Western blotting (non-reducing conditions), and the protein concentration was determined as described above.

**SFR Experiments**—SFR experiments were conducted at 25 °C using HBS (10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20) as running buffer and for diluting all the species injected in Bio-Rad experiments. 3500 RU of anti-hRII antibody were attached to a CMS sensor chip surface using the standard amine coupling procedure and an anti-hRII antibody (20 μg/ml) in 10 mM acetic acid (pH 4.0). TGF-β1 surfaces and control surfaces were prepared as described previously (16).

**Injections of RIIED-K5 or Equimolar Mixtures of RIIED-K5 and RIIED-E5 over TGF-β1**—Experiments were carried out with a flow rate of 5 μl/min in the case of RIIED-K5 injections and 50 μl/min in the case of RIIED-K5/RIIED-E5 mixture injections. Concentrations of RIIED-K5 alone or mixed with equimolar concentrations of RIIED-E5 (0–50 nm) were randomly injected in duplicate over TGF-β1 and control surfaces. Regeneration of the sensor chip between injections was accomplished as described above.

**Data Preparation and Analysis**—Sensorgrams were prepared and globally fit using kinetic models available in the SRevolution® software package (16). The data were prepared by the “double referencing” method (21).

**Testing the Antagonistic Potency Using a Luciferase Assay**—Mink lung epithelial cells stably transfected with the PAI-1 promoter fused to the firefly luciferase reporter gene (MLEC) were a generous gift of Dr. D. Riklin (24). In vitro TGF-β1 (10 pm) signaling assays were performed and analyzed as described previously (16) in the presence of 1) RIIED-K5, RIIED-E5, or RIIED-K5/RIIED-E5 equimolar mixture at various concentrations, 2) MP-RIIED-K5 or MP-RIED-K5/RIIED-E5 equimolar mixtures at various concentrations, or 3) MP-RIIED-K5 at 150 nm preincubated with various concentrations of RIIED-E5.

**RESULTS**

We have previously produced and purified RIIED-E5 for immobilization on a biosensor surface (22). In this study, we designed and expressed two additional fusion proteins that were tagged with the K5 coil (RIIED-K5 and MP-RIIED-K5). Both fusion proteins were N-terminally myc-tagged for detection and C-terminally His- and K5 coil-tagged for purification and dimerization, respectively.

Silver staining and Western blots of SDS-PAGE showed that the purified RIIED-K5 fusion protein contained disulfide-bridged aggregates since bands with higher than expected molecular weights were detected under non-reducing but not under reducing conditions (Fig. 1, A and B). Silver staining under reducing conditions indicated that the RIIED-K5 fusion protein was relatively pure (data not shown). The aggregate forms of RIIED-K5 were then removed from the preparation using a Centriprep 30 device (Fig. 1, C and D). In the case of MP-RIIED-K5, SDS-PAGE silver staining and Western blotting showed that the MP-RIIED-K5 fusion protein was relatively pure and contained no aggregates (Fig. 1, E and F).

**Evidence for Coiled Coil-induced Ectodomain Dimer Formation**—We next tested whether the coil-tagged ectodomains were able to dimerize through E5/K5 interactions using an SFR-based biosensor (the Biacore). An antibody that binds to the extracellular domain of RII was coupled to the sensor chip surface. To demonstrate that RIIED-E5 and MP-RIIED-K5 can heterodimerize, the following series of experiments were performed. First, to evaluate whether there was any nonspecific binding of MP-RIIED-K5 to the anti-hRII antibody, MP-RIIED-K5 (200 nm) was injected over the antibody surface. MP-RIIED-K5 did not interact significantly with the antibody alone (0–500 nm) or mixed with equimolar concentrations of RIIED-E5 (0–150 nm) were randomly injected in duplicate over TGF-β1 and control surfaces. Regeneration between injections was accomplished as described above.

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TGF-β Receptor Ectodomains with Enhanced Antagonistic Potency

We then examined the effect of coiled-coil-induced homodimerization of RIIED on its binding to TGF-β1. Solutions containing equimolar concentrations of RIIED-K5 and RIIED-E5 (0–50 nM) were injected over the same TGF-β1 surface. The resulting sensograms are strikingly different from those obtained with monomeric RIIED-K5 (Fig. 3) and monomeric RIIED-E5 (22). This difference confirms that dimerization of the coiled-coil domains of the RIIED fusion proteins did occur and demonstrates that the dimer has a significantly slower apparent off-rate than the monomeric RIIED. Global analysis of the RIIED-K5/RIIED-E5 sensograms using a simple model gave a poor fit. More complex biological models were then used to globally fit the data. Since TGF-β1 is a covalent dimer and since we artificially dimerized RIIED through coiled-coil interactions, two mechanisms of binding can be envisioned. 1) The RIIED-K5/RIIED-E5 dimer binds to two TGF-β1 molecules on the biosensor surface at the same time (avidity model), or 2) one RIIED domain within the coiled-coil-induced dimer binds to one monomer of TGF-β1, followed by the interaction of the other RIIED domain with the other monomer within the same TGF-β1 dimer. This corresponds to an overall 1:1 (dimer-to-dimer) stoichiometry model with a rearrangement step after the initial binding (rearrangement model). The rearrangement model depicted the interaction best as indicated by the standard deviation of the residuals (Table II). This is in agreement with our previous results showing that this model was the best to represent the binding of TGF-β1 to the RIIED-Fc artificial dimer (16).

Additionally, since the same TGF-β1 surface was used both for monomeric and coiled-coil-induced dimeric RIIED injections, if the two kinetic models depicting the interactions between TGF-β1 and both forms of RIIED are adequate, the calculated amount of active TGF-β1 on the surface (a global parameter determined during fitting) should be the same. The amount of active TGF-β1 was determined to be 62.5 ± 2 RU when fitting the monomeric RIIED-K5 interaction with a 2:1 stoichiometry model. In the case of the RIIED-K5/RIIED-E5 data, we found that this amount was 61.7 ± 1 RU for the rearrangement model, 40 ± 3 RU for the simple model, and 145 ± 4 RU for the avidity model. This observation further supports the validity of the 2:1 stoichiometry model for the interaction of TGF-β1 with monomeric RIIED-K5 and the validity of the rearrangement model for the interaction of TGF-β1 with the RIIED-K5/RIIED-E5 dimer.

TGF-β1 Binding to MP-RIIED-K5/RIIED-E5—When we examined the effect of coiled-coil-induced homodimerization of RIIED on its binding to TGF-β1. Solutions containing equimolar concentrations of RIIED-K5 and RIIED-E5 (0–50 nM) were injected over the same TGF-β1 surface. The resulting sensograms are strikingly different from those obtained with monomeric RIIED-K5 (Fig. 3) and monomeric RIIED-E5 (22). This difference confirms that dimerization of the coiled-coil domains of the RIIED fusion proteins did occur and demonstrates that the dimer has a significantly slower apparent off-rate than the monomeric RIIED. Global analysis of the RIIED-K5/RIIED-E5 sensograms using a simple model gave a poor fit. More complex biological models were then used to globally fit the data. Since TGF-β1 is a covalent dimer and since we artificially dimerized RIIED through coiled-coil interactions, two mechanisms of binding can be envisioned. 1) The RIIED-K5/RIIED-E5 dimer binds to two TGF-β1 molecules on the biosensor surface at the same time (avidity model), or 2) one RIIED domain within the coiled-coil-induced dimer binds to one monomer of TGF-β1, followed by the interaction of the other RIIED domain with the other monomer within the same TGF-β1 dimer. This corresponds to an overall 1:1 (dimer-to-dimer) stoichiometry model with a rearrangement step after the initial binding (rearrangement model). The rearrangement model depicted the interaction best as indicated by the standard deviation of the residuals (Table II). This is in agreement with our previous results showing that this model was the best to represent the binding of TGF-β1 to the RIIED-Fc artificial dimer (16).

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the solid lines represent the global fit for the 2:1 stoichiometry model.

**TABLE I**

| Model          | RIIED-K5 | RIIED-E5 | RIED |
|----------------|----------|----------|------|
| $k_d$ (m$^{-1}$ s$^{-1}$) | ($6.4 \pm 0.4$) × 10$^{10}$ | ($2.13 \pm 0.1$) × 10$^8$ | ($1.19 \pm 0.07$) × 10$^6$ |
| $k_d$ (s$^{-1}$) | ($6.6 \pm 0.4$) × 10$^{-5}$ | ($1.8 \pm 0.2$) × 10$^6$ | ($4.5 \pm 0.1$) × 10$^{-3}$ |
| $K_d$ RIIED (nm) | n/a | ($1.5 \pm 0.2$) | 0.2 |
| $K_d$ RIIED (nM) | ($3.7 \pm 0.6$) × 10$^{-7}$ | n/a | 0.922 |
| S.D. of residuals | 6.177 | 1.080 | n/a |

**TABLE II**

| Model          | RIIED dimerized through coiled-coil interaction | RIIED-Fc rearrangement |
|----------------|-----------------------------------------------|------------------------|
| $k_d$ (m$^{-1}$ s$^{-1}$) | ($6.4 \pm 0.4$) × 10$^{10}$ | ($5.3 \pm 0.3$) × 10$^7$ |
| $k_d$ (s$^{-1}$) | ($6.6 \pm 0.4$) × 10$^{-5}$ | ($2.7 \pm 0.2$) × 10$^{-2}$ |
| $K_d$ RIIED (nm) | n/a | ($1.35 \pm 0.01$) × 10$^{-1}$ |
| $K_d$ RIIED (nM) | ($3.7 \pm 0.6$) × 10$^{-7}$ | 0.922 |
| S.D. of residuals | 6.177 | n/a |

* in m$^{-1}$ s$^{-1}$; **, in m; n/a, not applicable.

solely from the increase in the molecular weight of the dimer versus the monomer (a ~2-fold difference). Instead, it must result from an increased affinity of the coiled coil-induced dimer for TGF-β1.

Analysis of the Antagonistic Potency of Non-dimerized and Coiled Coil-Dimerized Ectodomains—The ability of non-dimerized RIIED-E5, RIIED-K5, and MP-RIIIE-K5 versus dimerized RIIED-E5/RIIED-K5 and MP-RIIIE-K5/RIIED-E5 to...
RIIED-E5 in the presence of 150 nM MP-RIIIED-K5 (data point RLU measurements. RIIED-K5, at a constant concentration of 150 nM, was preincubated with RIIED-E5, the resulting dimer was able to block signaling by 50% at 7.5 nM. When MP-RIIIED-K5 alone (open triangles), or in the presence of equimolar RIIED-E5 (open diamonds), or RIIED-E5/RIIED-K5 equimolar amounts (filled circles), or in the presence of equimolar amounts of E5 coil (open diamonds), or in the presence of equimolar amounts of RIIED-K5 (open circles). Each data point represents the average ± S.D. of three independent experiments. RLU, relative luminescence.

block TGF-β1 signaling was tested using mink lung epithelial cells stably transfected with a TGF-β-responsive luciferase reporter gene.

Neither RIIED-K5 nor RIIED-E5 at concentrations up to 30 nM was able to inhibit TGF-β1-induced receptor signaling (Fig. 4A). In contrast, RIIED that was dimerized by preincubating equimolar concentrations of the two coil-tagged versions of RIIED was able to block signaling by 50% at 7.5 nM.

Somewhat surprisingly, MP-RIIIED-K5 alone enhanced TGF-β1 signaling (a 2-fold increase at 270 nM, Fig. 4B). This enhancement effect was also present when MP-RIIIED-K5 was preincubated with equimolar concentrations of E5 coil, which will mask the K5 coil hydrophobic and charged residues, indicating that the effect was not due to the unpaired coil strand. In contrast, when MP-RIIIED-K5 was preincubated with equimolar amounts of RIIED-K5, the resulting dimer was able to block signaling with an IC₅₀ of 40 nM. When MP-RIIIED-K5, at a constant concentration of 150 nM, was preincubated with varying concentrations of RIIED-E5, we observed an IC₅₀ of 7 nM RIIED-E5 (Fig. 4B). This shift in the IC₅₀ suggests that the antagonistic potency of the MP-RIIIED-K5/RIIED-E5 dimer may be limited by the affinity of the coiled-coil interaction (see “Discussion”).

DISCUSSION

To study the effect of RIIED homodimer and RIIED/MP-RIIIED heterodimer formation, both on the kinetics of binding to TGF-β1 and on the potency to antagonize TGF-β1 signaling, we adopted the strategy of tagging both ectodomains at their C terminus with the E5 or the K5 coil to promote their dimerization through coiled-coil interactions (Fig. 1). The ability of the coils to mediate the dimerization of the extracellular domains was confirmed using SPR (Fig. 2). We then compared the kinetics of binding to TGF-β1 of coil-tagged non-dimerized RIIED-K5 and RIIED-E5 (22) with those of the RIIED-K5/RIIED-E5 homodimer by SPR. This technique was chosen since it had been used successfully to determine the kinetics and stoichiometries of binding of RIIED and RIIED-Fc to TGF-β (16). In the case of coil-tagged non-dimerized RIIED-K5 (Fig. 3A), global analysis of the data indicated that the model best representing the data was a 2:1 stoichiometry model and that as for the E5 tag (22), the presence of the K5 coil within the fusion protein did not significantly affect RIIED binding to TGF-β1 (Table I).

In contrast, in the case of the RIIED-K5/RIIED-E5 homodimer, the model that best described the interaction of the coiled-coil dimer with TGF-β1 was a 1:1 dimer:dimer stoichiometry model including a rearrangement step (Fig. 3B and Table II). We previously determined that the apparent Kᵣ for the interaction of RIIED-E5 with synthetic K5 coil was 0.5 nM (22). Assuming that the coiled-coil interaction, when occurring between RIIED-K5 and RIIED-E5, has the same affinity, it is possible to calculate the percent of total RIIED that would be dimerized when equimolar amounts of RIIED-E5 and RIIED-K5 are preincubated. Based on this calculation, we estimate that the percent of dimer varied from 80 to 91% for the range of concentrations used in Fig. 3.

This conclusion is strongly reinforced by our previous results with the covalent dimer RIIED-Fc, i.e. the same kinetic model was found to best depict the interaction of that dimer with TGF-β1 (16).

Even though the homodimers of RIIED, obtained either by fusion to the Fc portion of an antibody or through coiled-coil interactions, shared the same mechanism of binding, their kinetic and thermodynamic constants displayed some differences (Table II). Indeed, the TGF-β1 interaction with the non-covalent coiled-coil dimer was characterized by a significantly faster initial on-rate and a slightly faster off-rate as compared with the RIIED-Fc dimer. Small differences were also observed in the rearrangement step. From these kinetic constants, it is possible to calculate an apparent dissociation rate and affinity (kₐ app and Kᵣ app), which globally describe the binding. This calculation yields similar apparent dissociation rates for both dimers (1.8 and 3.2 × 10⁻⁷ s⁻¹ for the coiled-coil versus Fc-induced dimers, respectively) but apparent Kᵣ that differ by 40-fold (1.5 and 60 nM for the coiled-coil versus Fc-induced dimers, respectively; see Table II). This difference results mainly from the difference in the initial on-rates.

A comparison of non-dimerized MP-RIIIED-K5 and MP-RIIIED-K5/RIIED-E5 heterodimer binding to TGF-β1 indicated that heterodimerization resulted in an increase in the apparent affinity (Fig. 3, C and D). We previously showed that RIIED and RIIED bind to TGF-β1 at independent sites (16). Therefore, within the MP-RIIIED-K5/RIIED-E5 heterodimer, both RII and RIII binding sites should be able to interact with...
one TGF-\(\beta\)1 dimer, thereby increasing the affinity by a multiple contact mechanism similar to that of homodimerized RIIED.

We complemented our kinetic study by testing the antagonistic potency of the non-dimerized and coiled coil-dimerized ectodomains using a luciferase reporter assay in mink lung epithelial cells (Fig. 4). The absence of any antagonistic effect with non-dimerized coil-tagged RIIED is in good agreement with previous results from our laboratory and from others, which showed that non-tagged RIIED has no effect upon signaling at concentrations as high as 500 nM (16, 18). We previously demonstrated that a form of RIIED artificially dimerized through the Fc portion of an antibody blocks signaling with an IC\(_{50}\) of \(-0.1\) nM (16, 18). We show here that RIIED that was homodimerized through coiled-coil interactions was able to block TGF-\(\beta\)1 signaling with an IC\(_{50}\) of 7.5 nM (Fig. 4A). This difference can be explained by the affinity of the coiled-coil interaction. That is, the potency of the coiled-coil RIIED dimer is likely limited by the affinity of the coiled-coil interaction since the \(K_d\) for the dimerization of the coil-tagged RIIEDs (0.5 nM, see above) is higher than the IC\(_{50}\) of the coherent Fc dimer (0.1 nM). In other words, at concentrations below 0.5 nM, a large portion of the coil-tagged RIIED population will be acting as a monomer rather than a dimer.

Non-dimerized MP-RIIIE5-K5, at concentrations higher than 150 nM, strikingly enhanced TGF-\(\beta\)1 signaling (Fig. 4B). Similar observations were made by Fukushima et al. (9) using a MP-RIIIE5 construct similar to ours in a \([3H]\)thymidine incorporation assay. This result contrasts with the antagonistic behavior of full-length RIIED (16, 26) and underlines the complexity of RII function. This agonist effect may result from the MP-RIIIE5 domain being able to interact with TGF-\(\beta\) in such a way that TGF-\(\beta\) is presented more effectively to RII on the cell surface. This effect also emphasizes the importance of understanding the mechanisms of interaction of the ectodomains when using them as a basis to design TGF-\(\beta\) antagonists.

In contrast to the MP-RIIIE5-K5 enhancement of TGF-\(\beta\)1 signaling, we observed an antagonistic effect for the coiled-coil-induced MP-RIIIE5-K5/RIIIE5-E5 heterodimer (Fig. 4B). As with the RIIED coiled-coil-induced homodimer, the antagonistic potency of the MP-RIIIE5-K5/RIIIE5-E5 mixture appeared to be limited by the affinity of the coiled-coil interaction. That is, when the concentration of MP-RIIIE5-K5 was held at a constant relatively high level with the concentration of RIIIE5-E5 varying, the IC\(_{50}\) was lower as compared with when the concentration of both MP-RIIIE5-K5 and RIIIE5-E5 varied across the curve (Fig. 4B). This lower IC\(_{50}\) likely results from a larger portion of the receptor ectodomain population being in a dimeric form.

The antagonistic potency of the coiled-coil MP-RIIIE5-K5/RIIIE5-E5 heterodimer can be explained by the ability of the RIIIE5 moiety to mask the RII binding site within TGF-\(\beta\)1, thereby preventing binding to the cell surface RII. We propose that the MP-RIIIE5-K5 moiety, within the heterodimer, acts to stabilize the TGF-\(\beta\)1/MP-RIIIE5-K5/RIIIE5-E5 ternary complex. This idea is supported by our SPR experiments, which showed that the MP-RIIIE5-K5/RIIIE5-E5 interaction with TGF-\(\beta\)1 was characterized by a complex wash-off phase, which included a slow dissociating component, as compared with non-dimerized RIIED (compare panels A and D of Fig. 3). Similarly, in the case of the coiled coil-induced RIIIE5-K5/RIIIE5-E5 homodimer, both ectodomains within the dimer simultaneously bind to the two monomers within the TGF-\(\beta\)1 dimer, resulting in a stable TGF-\(\beta\)1/RIIIE5-K5/RIIIE5-E5 ternary complex (Fig. 3B).

In conclusion, we have shown that coiled coil-induced dimerization can be used as a strategy to enhance the potency of TGF-\(\beta\) receptor ectodomains to sequester TGF-\(\beta\) and inhibit signaling. The main advantage of this coiled coil-induced dimerization strategy is that well defined monomeric, homodimeric, and heterodimeric forms of the receptor ectodomains can be rapidly produced and evaluated. The disadvantage of this approach resides in the fact that the potency of the coiled coil-induced dimer is limited by the affinity of the coiled-coil interaction. However, this can be overcome by modifying the coiled-coil sequence such that a disulfide bond is formed when the coil strands interact.

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