Structural Basis for the Binding Specificity of Human Recepteur d’Origine Nantais (RON) Receptor Tyrosine Kinase to Macrophage-stimulating Protein*

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Background: RON and MET receptors bind their ligands MSP and HGF selectively and activate different signaling pathways. Crystallographic and analytical ultracentrifugation studies provide important information about RON-MSP interaction. Signaling pathways must be exquisitely regulated with no cross-reactivity between related systems.

Ron receptor tyrosine kinase (also known as hepatocyte growth factor receptor) is a type 1, single-pass membrane-spanning cell surface receptor for macrophage-stimulating protein (MSP). RON and the proto-oncogene MET are the only members of the Class VI receptor tyrosine kinase family, sharing ~64% sequence identity within their cytoplasmic kinase domains and ~33% identity within their ligand-binding ectodomains (Fig. 1A). RON is widely expressed in macrophages, epithelial tissues, adenocarcinoma cells, bronchial epithelial cells, granulocytes, and monocytes (1–3). It functions in the MSP-mediated inflammatory activities under cellular stress conditions and in the innate immune responses to bacterial infections (4–6). High levels of RON are detected in patients with ulcerative colitis, deep endometriosis, and several types of epithelial cancers, implicating RON in the progressions and pathogenesis of these diseases (7, 8). Multiple alternatively spliced variants of RON regulate cancer metastasis (7, 9–17). In addition to MSP, RON also forms complexes with MET, plexin receptor B1–B3, β1 integrin, and epidermal growth factor receptor to control cellular migration and invasion processes (18). RON disrupts the plexin-integrin β4 complex, which regulates the MSP-dependent migration of pancreatic cancer cells (19). RON also interacts with several hyaluronan-binding proteins, including CD44v6, RHAMM, and hyaluronidase 2 (20).

The abbreviations used are: RON, recepteur d’origine nantais; MET, MET receptor tyrosine kinase; MSP, macrophage-stimulating protein; HGF, hepatocyte growth factor; Sem, semaphorin domain; PSI, plexin-semaphorin-integrin domain; IPT, immunoglobulin-plexin-transcription factor domain; SPI, Sema-PSI-IPT1–4; AUC, analytical ultracentrifugation; SV, sedimentation velocity; SE, sedimentation equilibrium; RMsd, root mean square deviation; SP, serine protease; N domain, N-terminal hairpin domain; PDB, Protein Data Bank; AU, absorbance units.

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The RON polypeptide comprises an extracellular ligand binding domain and a cytoplasmic tyrosine kinase domain, connected by a short membrane-spanning region. The RON ectodomain is subdivided into six domains: the N-terminal semaphorin domain (Sema), a small cysteine-rich plecin-semaphorin-integrin domain (PSI), and four immunoglobulin-plecin-transcription factor domains (IPT1–4). Precursor RON is a single-chain glycosylated protein that undergoes proteolytic maturation at a consensus furin cleavage site (Arg309–Gly310 in the Sema) prior to translocation onto the cell surface (1). The mature receptor consists of a 40-kDa RON α-chain containing the N-terminal half of Sema and a 145-kDa RON β-chain containing the rest of the protein. Currently, the MSP-mediated activation of the RON receptor is presumed to be similar to the proposed activation mechanisms of MET by its ligand, HGF, a protein homologous to MSP (25). In other words, the binding of mature MSP (comprising a disulfide-linked α- and β-chain heterodimer, hereafter termed MSPαβ) to the RON ectodomain initiates the formation of a signaling-competent RON dimer on the cell surface, juxtaposing the cytoplasmic kinase domains sufficiently close to induce autophosphorylation of conserved tyrosine residues, which leads to downstream signal transduction (4, 26, 27). The ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), focal adhesion kinase, and β-catenin molecules are activated by the MSP-mediated RON signal transduction pathways (28, 29).

The RON-specific ligand is the MST1 gene product, MSP. The 80-kDa serum growth factor is composed of six domains: the N-terminal hairpin domain (N domain), four Kringle domains (K₁–K₄), and a chymotrypsin-like serine protease (SP) domain that is devoid of a catalytic triad (replaced by Gln522, Gln568, and Tyr661) (30). MSP shares ~50% sequence identity with HGF (Fig. 1B), and both ligands belong to the plasminogen-like growth factor family (31). Circulating MSP is synthesized in liver cells as a single-chain precursor (pro-MSP) that does not bind to RON (32, 33). Under cellular stress, pro-MSP undergoes proteolytic maturation to become a disulfide-linked MSPαβ, which binds and activates RON (33). Several serine proteases (kallikreins, matriptase, hepsin, and human airway trypsin-like protease) recognize the cleavage site (Arg483–Val484) between the K₄ and SP domains. The 50-kDa α-chain (MSPα) contains the N and K₁–K₄ domains, whereas the 30-kDa β-chain (MSPβ) comprises the SP domain (4, 30, 31). Both α- and β-chains of MSP are essential for its biological activity; however, the receptor-specific binding to RON Semada mediated by the MSP β-chain alone (33–35). Mutagenesis studies identified the interacting residue pair, Arg682/Glu648 and the neighboring Arg683 in MSPβ as critical for RON receptor binding and activation (32–34, 36). The interaction between MSPα and RON is weak and not always detectable (33, 34). By contrast, HGFα binds to the MET with an affinity higher than that of HGFβ (37). In fact, splice variants of HGF, NK₁ and NK₂, function as MET agonist and antagonist, respectively (38). To gain insights into the structural determinants of RON-MSP specificity, the crystal structure of the RON SPI₁-MSPβ complex was determined at 3.0 Å, and the binding interaction was characterized using analytical ultracentrifugation (AUC).

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Protein Purification—The RON ectodomain was amplified from pMSCVneo-hRON-2HA (kindly provided by Dr. Pamela A. Hankey, Pennsylvania State University). This human MSTIR gene included the single nucleotide polymorphism resulting in a R322Q mutation. The purified proteins from the conditioned Drosophila melanogaster Schneider 2 (S2) medium are glycosylated at predicted sites as described previously (39, 40). RON Semada (Glu25–Gly310), SPI₁ (Glu25–Gly683), and SPI₁–₄ (Glu25–Ser956) contain two N-terminal residues (Arg23 and Ser24) and eight C-terminal residues (Thr684, Gly685, and His686–His691) from the expression vector. The furin cleavage site in the RON Semada (KRRRGA) was mutated to a thrombin cleavage site (KLVRGSP) (39).

The human MST1 gene was amplified from a cDNA clone (ID 5190966, Open Biosystems, Inc.). The MSP proteins, containing a C-terminal His₁₀ tag, were purified from the D. melanogaster S2 conditioned medium as previously described (40). MSP (Gln19–Gly711), MSPα (Gln19–Lys466), and MSPβ (Phe465–Gly711), which includes 19 linker residues to the α-chain to facilitate five physiologically relevant disulfide bonds (40, 41)) were stored in 20 mM MES, pH 6.0, 0.1 M NaCl, 0.02% (v/v) sodium azide at ~80 °C. MSPβ used in crystallization studies also contained a C672S mutation, introduced to prevent an incorrect disulfide bond formation between Cys672 and Cys808 and to maintain the Cys668–Cys808 linkage (41). The MALDI-TOF analyses of MSPα (52,167 Da, ε280 = 85,510 M⁻¹ cm⁻¹), MSPβ (28,295 Da, ε280 = 48,470 M⁻¹ cm⁻¹), and MSPαβ (79,266 Da, ε280 = 139,430 M⁻¹ cm⁻¹) gave molecular masses of 54,022 ± 68, 29,210 ± 19, and 82,137 Da, respectively. Higher experimental molecular masses than the calculated values (MSPβ ΔMM = 915 Da, MSPα ΔMM = 1,855 Da, and MSPαβ ΔMM = 2,871 Da) are consistent with N-glycosylations at Asn615 for MSPβ, at Asn72 and Asn296 for MSPα, and at all three sites for MSPαβ. Pro-MSP and single-chain MSPβ were cleaved at the Arg183–Val84 peptide bond by treatment with the catalytic domain of human matriptase-1 at a 1:16,000 enzyme/substrate ratio in 50 mM Tris-HCl, pH 8.0 at 2 °C. The serine protease inhibitor mixture (Sigma) was added to terminate proteolysis, and the protease was removed by passing the reaction mixtures through the benzamidine-Sepharose 4 (FF) resin (GE Healthcare). Flow-through fractions were analyzed by SDS-PAGE under reducing and non-reducing conditions to confirm a complete conversion of pro-MSP into MSPαβ.

Crystallization, Data Collection, and Structure Determination—Crystals of RON SPI₁ in complex with MSPβ were obtained at room temperature using the vapor diffusion method. MSPβ and RON SPI₁ at an ~1:1 molar ratio were mixed to yield ~60 μM concentration. The drops comprised equal volumes of SPI₁-MSPβ and mother liquor containing 0.1 M Tris-HCl, pH 8.5, 20% (w/v) PEG 4000, 8% (v/v) isopropyl alcohol. The crystallization conditions were identified using sitting drop 24-well screens (Hampton Research). Optimized conditions were generated by a systematic variation of these parameters.

Crystals were grown at 20 °C for 16 days using 2 μL drops comprising 0.1 M glycine, pH 7.5, 20% (v/v) PEG 4000, 8% (w/v) isopropyl alcohol. The crystals belong to space group P3₁, with unit-cell parameters a = b = 139.4 Å, c = 85.5 Å, and V = 26,342 Å³. The crystals were cryoprotected in a solution of 0.1 M glycine, pH 7.5, 20% (v/v) PEG 4000, 8% (w/v) isopropyl alcohol with 22% (v/v) glycerol. X-ray diffraction data were collected at 100 K on a Rigaku MicroMax-0000 rotating anode X-ray generator equipped with a Rigaku R-AXIS REX II detector and a PerkinElmer III area detector, respectively. The data were processed and reduced using MOSFLM/OSPI (42, 43). The crystallographic statistics are given in Table 1. The crystallographic R-factors were 56.5% for the native data set and 24.8% for the anomalous data set. The MAD intensity data were processed by the FLASPH program package (44). The molecular replacement model was generated using MOLREP (45). The full model was refined using REFMAC (46) and Coot (47). The final model has a resolution of 2.1 Å with an R-factor of 20.9% for 1,216,006 observed reflections (R₁ = 26.8% for 1,192,341 reflections including the anomalous scattering data, 26.5% for 1,130,908 reflections excluding the anomalous scattering data). The final model consists of 1,513,122 atoms with 20 water molecules. The model was validated using PROCHECK (48), Molprobity (49), and WHAT_CHECK (50). The structural superposition of the RON SPI₁-MSPβ complex with the RON SPI₁-HGF complex (PDB ID 4GBO) yielded a root mean square deviation of 0.06 Å in all non-hydrogen atoms. The crystallographic refinement statistics are given in Table 1.
alcohol, and 4% (v/v) polypropylene glycol 400 (derived from condition 41 of Hampton Crystal Screen I). For data collection, thin plate-shaped RON SPI₁–MSPβ crystals were transferred to mother liquor supplemented with 30% (v/v) glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected at the General Medicine and Cancer Institutes Collaborative Access Team microbeamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL), which was equipped with a MARmosaic CCD detector. Diffraction data were processed with XDS to a resolution of 2.8 Å (42). The structure was determined by molecular replacement (in the space group P2₁2₁2₁) using the program PHASER (43) with the free RON Sema (PDB code 4FWW) and free MSPβ (PDB code 2ASU) structures as the search models (39, 41). Problems with the progress of the refinement due to pseudomerohedral twinning were tracked with the programs SFCHECK and XTTRIAGE in PHENIX (44, 45). Structure refinement, including a pseudomerohedral twinning rule, was conducted at 3.0 Å resolution using the program REFMAC5 (46). Model building and structure modification was performed using the interactive computer graphics program COOT (47). Molecular interfaces were calculated using PISA and ProFace (48, 49). Topological complementarity of interacting surfaces was calculated using the shape correlation statistic program SC (50), as implemented in CCP4. Figures was performed using the interactive computer graphics program RASTER3D (51, 52).

Analytical Ultracentrifugation—Sedimentation velocity (SV) and sedimentation equilibrium (SE) experiments were performed at 20 °C using a ProteomeLab Beckman XL–A ultracentrifuge with an absorbance optical system and a 4-hole An60-Ti rotor (Beckman Coulter). For SV, 400 μl of protein, dialyzed in PBS, pH 7.4, and 420 μl of PBS were loaded into the sample and reference sectors of the dual-sector charcoal-filled epon centerpieces. The cells were centrifuged at 50,000 rpm, and the absorbance data for 0.125–30 μM proteins were collected at 230, 250, or 280 nm to obtain linear signals of <1.25 absorbance units. The absorbance signal was monitored in a continuous mode with a step size of 0.003 cm and a single reading per step. Sedimentation coefficients were calculated from SV profiles using the program SEDFIT (53). The continuous c(s) distributions were calculated assuming a direct sedimentation boundary model using the Lamm equation with maximum entropy regularization at a confidence level of 1 S.D.

For SE, the sample sector of dual-sector centerpieces was filled with 140–180 μl of protein (0.5–14 μM), and the reference sector was filled with 150–190 μl of PBS. Each SE experiment was conducted at three or four speeds (8,000–24,000 rpm) at 20 °C, increasing from the lowest to the highest speed. SE experiments of SPI₁–MSPαβ association were determined at 4 °C because analysis of the SV experiments, followed by SDS-PAGE, indicated occasional limited degradation of the protein mixture at 20 °C. Equilibrium was considered as reached when the RMSD value of successive scans taken at 3-h periods was below the noise level as determined by SEDFIT. Absorbance was scanned at a wavelength interval of 0.001 cm with 20 replicates/step. The SE curves were analyzed using the non-linear regression analysis program SEDPHAT to obtain the K_D, based on the Boltzmann distributions of ideal species in the centrifugal field (54). The SDS-PAGE and Western blotting assays under non-denaturing and denaturing conditions were used to evaluate protein integrity at the end of SV and SE experiments. The density and viscosity of buffers at 20 °C and 4 °C were calculated using SEDNTERP (55). The partial specific volumes of glycosylated proteins were calculated as published (56). The structure-based hydrodynamic properties of proteins were calculated using the bead shell-modeling program HYDROPRO (57). The c(s) distributions and SE profiles were prepared with the program GUSSI (C. A. Brautigam, University of Texas Southwestern Medical Center).

RESULTS AND DISCUSSION

The RON-MSP interaction was investigated using biophysical and structural approaches to shed light on the receptor-ligand specificity in this and related systems. The characterization of moderately to strongly binding complexes provided explanations for the MSP specificity to RON relative to other receptors, which support and expand our understanding of RON signaling from previous investigations (32–36, 40, 41, 58). The crystal structure revealed the detailed receptor-ligand interactions within a 1:1 complex. The AUC analysis examined the stoichiometry of the interaction between RON SPI₁ and MSPβ at lower concentration than that used in the crystallization, and also examined the interaction with the full-length mature MSPαβ. The AUC showed the same 1:1 stoichiometry for the RON SPI₁–MSPβ complex as in the crystals. In contrast, in the presence of MSPαβ, the majority of the complexes exhibited the 1:1 stoichiometry but also revealed a minor higher stoichiometry species, relevant to the physiological function of RON in signal transduction.

Structure Determination—Data processed at 2.8 Å resolution in space group P222 showed systematic intensity absences along all principle axes, consistent with space group P2₁2₁2₁. However, the intensity statistics indicated a possible twinning by pseudomerohedry (I = 0.621, L test = 0.377). Molecular replacement in space group P2₁2₁2₁ identified a single RON Sema and a single MSPβ with Z-scores for the rotation and translation functions of RFZ = 10.1 and TFZ = 19.2 for Sema, and RFZ = 6.8 and TFZ = 28.2 for MSPβ. Thus, refinement commenced using the data processed in the P2₁2₁2₁ space group. Once it became clear that the refinement was not progressing, the data were reprocessed in space group P1, yielding unit cell dimensions of a = 63.9 Å, b = 107.1 Å, c = 147.5 Å, α = 90.1°, β = 90.1°, γ = 90.1° (i.e. all crystal cell angles were close to 90°). Next, the 2.8 Å resolution data were processed in space group P2₁, using each of the principle orthorhombic cell axes as the potential unique monoclinic b axis. The resulting three R_merge values were 0.195, 0.202, and 0.212 for the choice of the orthorhombic unit cell a, b, and c, respectively. The high R_merge values may be attributed to the decrease in diffraction intensity below 3.0 Å resolution (∣I/óI∣ < 1.5). All three data sets yielded molecular replacement solutions with two complexes in the asymmetric unit, which exhibited non-crystallographic 2-fold screw symmetry along the corresponding non-unique crystal axes. Refinement was carried out at 3.0 Å resolution using the data sets that yielded the two better Rmerge values. The correct unit cell choice was determined to be a = 106.6 Å, b = 63.8 Å, c = 146.0 Å, α = 90.0°, β = 90.1°, γ = 90.0° based on the packing
of molecules in the asymmetric unit. The correct crystal cell parameters exhibited identical RON SPI1–MSPβ interfaces of the two complexes in the asymmetric unit. In contrast, one of the complexes in the incorrect choice of unique unit cell axis had small but systematically longer distances between interacting receptor and ligand residues. Presumably, this distortion was introduced by the incorrect assignment of two unit cell angles, the one assigned exactly 90° and the second slightly different from 90°. It should be emphasized that the structure of individual molecules remained the same in both the correct and incorrect cell units; only the packing of the molecules in the crystals was subtly different. Table 1 provides the data processing and refinement statistics.

Finally, through the entire study period, extensive attempts to improve the crystals were unsuccessful. Nevertheless, the quality of the structure reported here is sufficient to shed light on the biologically important questions.

Structure of RON SPI1.—The biologically active MSP is generated by proteolytic cleavage at Arg483–Val484 in the linker region between the α- and β-chains, yielding the disulfide-linked MSPαβ. The single-chain MSPβ construct used in the crystallization included the uncleaved 19-amino acid linker region (Cys468–Arg483) between the α- and β-chains, ensuring that all of the cysteine residues in MSPβ form disulfide bonds. Previous SPR studies showed that this single-chain MSPβ exhibited similar binding affinities to immobilized RON ectodomain variants of increasing length (Sema, SP, SPI1, and SPI4), indicating that only the RON Sema contributes to the binding affinity between MSPβ and RON ectodomain (40). MSPαβ SPR binding experiments to the immobilized RON ectodomain constructs produced the same result.3 This is consistent with the lack of strong binding between MSPα and RON ectodomain (33, 34, 40).

The association of single-chain MSPβ with SPI1 was sufficiently tight to yield crystals of the complex, but our attempts to obtain crystals of two-chain MSPβ (cleaved at Arg483–Val484) with either single- or two-chain SPI1 were unsuccessful. In retrospect, the uncleaved regions of MSPβ and SPI1 are involved in crystal contacts, and the cleavages in these loops might have prevented the formation of these crystal-packing interactions. Table 1 summarizes the crystallographic data for the SPI1–MSPβ structure. The RON SPI1 model includes residues Gln28–Glu483. No electron density is associated with RON residues 25–27 at the N terminus; residues 358–360 of Sema; and residues 582–583, 598–602, 621–633, and 646/647–651 of IPT1, and these are omitted from the model. The electron density map revealed N-glycosylation at one of the five predicted sites on SPI1, enabling model building of a GlcNAcβ(1,4)GlcNAc unit at Asn488 (Sema) of one molecule in the asymmetric unit and a Manβ(1,4)GlcNAcβ(1,4)GlcNAc of the second Asn488 in the asymmetric unit. For the free RON crystal structure, the cleavages of 17 N-terminal amino acids and the C-terminal half of IPT1 occurred under the crystallization condition (39). When bound to MSPβ, both of these regions remained intact (Fig. 2A), providing the first view of the RON IPT1 domain and its spatial relationship to the Sema and PSI domains. An interdomain disulfide bond between the N-terminal Cys29 of Sema and Cys590 of IPT1 tethers the Sema, PSI, and IPT1 domains and restricts domain flexibility (Fig. 2A). The intact N-terminal polypeptide meanders adjacent to the β-strands 6D and 6E of Sema, disrupting the hydrogen bond interactions between these β-strands, as observed previously in the free RON SP structure (39). In contrast, MET lacks an equivalent interdomain Cys29–Cys590 disulfide bond despite the amino acid sequence conservation (Cys26 and Cys548 in the MET numbering system) (59). Instead, Cys548 is disordered, and Cys548 is unpaired in the MET SPI2/lnB complex structure (59). Niemann (25) had suggested an alternative interdomain disulfide bond between Cys26 and the non-conserved Cys800 of MET IPT3. Because RON IPT3 does not have an equivalent cysteine, this Cys29–Cys590 disulfide bond is proposed as the physiologic interdomain linkage for RON. In addition, the cysteine residue pattern differs in the distinct extrusion regions of RON and MET Semas, resulting in two disulfide bonds in RON and one disulfide bond in MET (Fig. 1A). The remaining 22 cysteine residues of RON and MET SPI1 form conserved intradomain disulfide linkages (39). In the free RON SP structures, the β4D–β4D′ Sema loop containing the proteolytic maturation site is disordered in both intact and cleaved proteins (39). This β4D–β4D′ loop adopts a defined conformation in the complex, stabilized by crystal contacts. The physiologic relevance of this loop conformation is uncertain because of its involvement in crystal contacts and the introduction of mutations that replaced the furin-specific sequence by a thrombin cleavage sequence.

Comparison of the free and MSPβ-bound structures reveals that the RON PSI motif is oriented differently with respect to the Sema (Fig. 2B). This may be significant because PSI motifs typically serve as linkers that orient the flanking domains for

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3 N. V. Gorlatova and O. Herzberg, unpublished results.
interactions with different partner proteins (60). Previously, rigid body rotations of PSI with respect to Sema have been noted in MET SP-HGF and MET SPI2-InlB structures, in which the PSI relative orientations differed by 60° (59). Rigid body rotation analysis of the two RON structures, using the program DynDom (61), showed a 45° rotation around an effective hinge axis running parallel to residues 525–527 connecting the Sema and PSI domains (Fig. 2B). This domain motion resulted in the closure of the Sema-PSI interface, doubling the buried surface area upon closure from 385 Å² in the free RON SP structure to 820 Å².

The 17 N-terminal residues, previously missing in the RON SP structure, also contribute to the Sema-PSI contacts (Fig. 2A). In light of the conformational restriction imposed by the Cys29–Cys590 interdomain disulfide bond and the contacts between the N-terminal region of Sema and IPT1, it remains to be seen whether and how the PSI motif still functions in the mechanism as a hinge that induces tyrosine phosphorylation at the RON cytoplasmic kinase domain following MSP binding to the ectodomain.

RON IPT1 belongs to the early Ig-like (E-set) IPT/TIG domain superfamily (Figs. 1A and 2C). Members of this superfamily usually mediate protein-protein interactions. The six core β-strands of IPT1, arranged in the order ABE and CFG, form an antiparallel two-layer sandwich. Despite their low (20%) amino acid sequence identity, DALI analysis (62) revealed that the RON and MET IPT1 are the closest structural

![Figure 1. Structure-based sequence alignments of human RON SPI1 and MSPβ with their respective family members MET SPI1 and HGFβ. Identical residues are colored red, and cysteines are colored gold. Matching colored symbols indicate pairs of cysteines that form disulfide bonds. This figure was prepared with ESPript 3.](image-url)
homologs (PDB code 2UZX, Z = 9.5, RMSD = 2.5 Å, for 80 paired Ca atoms) (Fig. 2C). Superposition of the Semas of RON SPI1 and MET SPI1 structures showed an ~16-Å shift in the positions of the respective IPT1 domains (Fig. 2D). These structural differences may contribute to their functional specificities. For example, the region surrounding the β1B’-β1B” hairpin loop of MET IPT1, which is the primary ligand binding site for the bacterial invasion protein, InlB, which uses MET as a specific cell surface receptor (59). The analogous RON IPT1 region differs in both sequence and size from that of MET in that it is larger than MET by 21 amino acids, which are inserted into the N-terminal residues of the MET IPT1 domain, introducing only minor conformational changes, primarily in loop regions (35). In addition, the RONΔ110 splice variant, which comprises only part of the IPT1 followed by IPT2–4 and the cytoplasmic kinase domain, also exhibits constitutive transphosphorylation activity (11, 12). Moreover, the RONΔ15/6 splice variant, encoding a 20-amino acid insertion in the IPT1 domain, introduces another level of functional regulation by proteolysis. This variant requires MSP binding for activation, but cleavage within the inserted region generates the constitutively active RONΔ110. Together, these constitutively active RON variants suggest that IPT1 plays a role in regulating ligand-dependent dimerization of the receptor.

Structure of MSPβ—For consistency, we follow the structural unit assignments previously used to describe the structure of two-chain MSPβ, which included the 19-amino acid linker region cleaved at Arg483-Val484 (41). As the two-chain MSPβ, the single-chain MSPβ adopts the classic chymotrypsin-like serine protease fold (Fig. 2A). There is no electron density for the N-terminal residues of the αβ linker residues 465-467, residues 545-548, and the entire L8 loop (residues 608-615/616, including the N-glycosylation site at Asn615; Fig. 1B). Superposition of the single- and two-chain MSPβ structures reveals only minor conformational changes, primarily in loop regions (L4, L5, L10, L11, and L13), yielding RMSD of 0.7 Å for 205 paired Ca atoms (Fig. 2E). However, there is a dramatic conformational change associated with the proteolytic cleavage at Arg483-Val484, resulting in the rearrangement of the 19-residue αβ linker region. The N-terminal residue Val484, generated from the cleavage at the Arg483-Val484 peptide bond, inserts into a pocket buried under the L8 loop (41). By contrast, the
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intact linker in the single-chain MSPβ is fully solvent-exposed and interferes with the placement of the L8 loop, leading to its disorder (Fig. 2E).

RON SPI1-MSPβ Interface—There are two RON SPI1-MSPβ complexes in the asymmetric unit, and the alignment of the two copies yielded an RMSD of 0.17 Å for 861 paired Ca atoms. The superimposing receptor-ligand binding interface spans the β3A-β3B, β3C-β3D, and β4C-β4D loops of SemA and the α1, L4, L6, L10, L11, and L13 regions of MSPβ (Figs. 1A (4 and B) and 3 (A and B)). Using the program PISA (48), the average buried surface areas of the two complexes in the asymmetric unit are ~898 and ~874 Å² for SemA and MSPβ, respectively. The total buried surface area of ~1770 Å² engages 26 or 28 residues of the 2 SemA molecules in the symmetric unit and 25 or 26 residues of the 2 MSPβ molecules. The molecular contacts include multiple salt bridges and hydrogen bonds as well as hydrophobic and van der Waals interactions (Fig. 3A). The RON SemA-MSPβ interface involves the MSPβ Arg683 residue previously identified by the site-directed mutagenesis studies as essential for RON receptor recognition (34). It is also consistent with the prediction by Carafoli et al. (41) based on the homology to the MET SP-HGFβ structure (58).

The degenerate serine protease active site cleft of MSPβ comprises the center of the receptor recognition surface, with the protruding β3A-β3B hairpin loop of SemA inserted into the MSPβ cleft (Fig. 3A). Two MSPβ arginine residues (Arg521 and Arg683) in the β-barrel subdomains flanking the serine protease cleft are embedded in the receptor-ligand interface (Fig. 3A). Conversely, the glutamic acids on SemA complement the buried positive charges on MSPβ (i.e. RON Glu287 and Glu289 in the vicinity of MSP Arg521 and RON Glu190 in the vicinity of MSP Arg683). In addition, MSP Arg521 interacts with the backbone O of RON Pro288 (not shown), and Arg683 interacts with RON Ser195. RON Glu287 also interacts with the hydroxyl groups of MSP Ser565, and RON Glu289 interacts with the backbone NH group of MSP Cys527 (not shown). Another charge-charge interaction occurs between RON Arg220 and MSP Glu658 at the center of the interface. In earlier mutagenesis studies, the replacement of MSP Arg683 with a glutamine abolished its binding ability to cells expressing RON receptor (34), supporting our conclusion that the RON-MSP interface in the crystal structure is physiologically relevant. Moreover, the NH2 group of RON Glu190 interacts with the carboxylate groups of MSP Glu644 as well as with the backbone carbonyl of MSP Arg639 (the latter is not shown). These molecular contacts suggest that the buried Glu193 of RON-Sema plays a crucial role in ligand recognition. In addition to interacting with MSP Arg683, the carboxylate group of RON Glu190 forms a salt bridge with the guanidinium group of MSP Arg639, located at the interface periphery (Fig. 3A). Finally, an aromatic interaction occurs between the side chains of two histidine residues, RON His424 and MSP His528. These histidines are probably uncharged because the crystals were obtained at pH 8.5. Their imidazole groups stack face-to-face at the periphery of the Sema-MSPβ interface, similar to interactions found in other crystal structures (63).

Structural Basis for Receptor-Ligand Specificity in RON-MSPβ and MET-HGFβ—Despite the common recognition surfaces, the RON-MSPβ and MET-HGFβ interfaces differ in details, which explains the unique specificity and lack of cross-reactivity of these binding partners. The locations of the receptor-ligand interfaces in RON SPI1-MSPβ and MET SP-HGFβ are approximately the same (Fig. 3, B–D). Both interfaces bury a total of ~1700 Å² of surface area involving ~50 amino acids. The local density for RON-MSPβ and MET-HGFβ complexes is also similar at ~37, calculated using the program ProFace (49). This value falls within the local density values of 42 ± 6, reported for specific protein-protein interfaces (64, 65). Superposition of the MSPβ and HGFβ in the two complex structures highlights the differences (RMSD of 0.97 Å for 182 paired Ca atoms) (Fig. 3E). The most striking feature is the projections of the β3A-β3B hairpin loops of RON and MET Sema into their respective ligands. Due to the different length and functionality of the amino acids, the RON β-hairpin (colored magenta in Fig. 3E) projects more deeply into the MSPβ cleft than the corresponding MET β-hairpin into the HGFβ cleft (colored green). The deeper projection of the RON β-hairpin loop may be attributed to its smaller amino acids (Gly192 and Gln193) compared with those on the MET β-hairpin (Asp190 and Arg191). The key discriminating amino acids on the respective ligands are MSPβ Gln568 and HGFβ Asp578 (Fig. 3E). MSPβ Gln568 would clash with MET Arg591, whereas MET Arg191 forms a salt bridge with the shorter Asp578 of its own ligand, HGFβ. Conversely, if the MET β-hairpin were to adopt the same conformation as RON β-hairpin, the side chains of MET Asp590 and Arg191, which are larger than their RON counterparts (Gly192 and Gln193), would clash with MSPβ Gln568 and the backbone side chain of Arg539.

Additionally, MSPβ contains two more residues (Ser526–Cys527) in its L4 loop when compared with the same loop of HGFβ (Fig. 1B). The Cys527 in MSPβ forms a disulfide bond with the Cys565 on β6, whereas such a disulfide bond is absent in HGFβ. Consequently, the L4 loops of MSP and HGF adopt entirely different conformations. The MSPβ L4 loop conformation enables the stacking of the MSPβ His528 against RON His124 (Fig. 3D, I) and an interaction of the backbone amide of Cys527 with the carboxylate group of RON Glu289 (not shown). This imidazole ring stacking may also be a RON-MSP selectivity determinant because RON His424 is located on the αEx2 helix of the extrusion region (residues 371–429). The structural integrity of the RON extrusion region is maintained by two adjacent disulfide bonds (Cys385–Cys407 and Cys386–Cys422) (Fig. 1A). By contrast, the extrusion loop of MET is partially disordered in both MET-HGFβ and MET-InIB structures (58, 59). Thus, the different folds adopted by respective extrusion regions of the MET and RON structures suggest distinct functional roles (39, 58).

Structure-based sequence alignments of MSPβ, HGFβ, and plasmin showed that MSPβ contains two clusters of triple arginine residues in the L10 (Arg637, Arg639, Arg641) and L13 (Arg683, Arg687, Arg689) loops (41). The authors proposed these arginine-rich regions as the specificity determinants of RON-MSP recognition. Of the 6 arginine residues, only Arg683 on MSPβ L13 is fully embedded in the interface (Fig. 3E), yet Arg683 is unlikely to be a specificity determinant because the HGFβ counterpart is also an arginine (Arg641). MSPβ Arg687 and Arg689 are located remotely from the RON-MSP interface. The
FIGURE 3. **Comparison of RON Sema-MSP\(\beta\) and MET Sema-HGF\(\beta\) interfaces.** A, stereoscopic representations of the RON Sema-MSP\(\beta\) interface residues (colored blue and yellow, respectively). The backbone interaction of MSP\(\beta\) with RON side chains and vice versa, including MSP Arg\(^{639}\) oxygen with RON Gln\(^{193}\), MSP Cys\(^{527}\) nitrogen with RON Glu\(^{289}\), and MSP Arg\(^{521}\) with RON Pro\(^{288}\) oxygen, are not shown for clarity. B, interface shape complementarity in the RON SPI1-MSP\(\beta\) complex. C, ribbon representation of superposed RON SPI1-MSP\(\beta\) (colored blue and yellow) and MET SP-HGF\(\beta\) (colored gray and dark gray; PDB code 2UZX) interfaces with the Semas as reference. D, interface shape complementarity in MET SP-HGF\(\beta\) complex (colored gray and dark gray; PDB code 2UZX). E, stereoscopic representations of superposed interface residues of RON SPI1-MSP\(\beta\) complex (colored blue and yellow) versus those of MET SP-HGF\(\beta\) complex (colored gray). Specificity determinant residues in RON and MET \(\beta\)3A-\(\beta\)3B hairpin loops are highlighted in magenta and green, respectively.
caveat is that MSP L13, including Arg^{687} and Arg^{689}, is involved in an interaction with a symmetry-related RON Sema that generates an entirely different Sema-MSPβ interface. This crystal contact also involves the intact B4D-B4D' maturation loop of RON and the uncleaved linker region of MSPβ, and therefore might not reflect interactions within the physiological complex. In addition, because the L13 loop is located on the same face of MSPβ as the αβ linker, it may mediate interactions between the α and β domains of MSP rather than interaction with RON. Nevertheless, the possibility of conformational transition of these arginine residues in the L13 loop upon binding to RON receptor in solution cannot be ignored. For L10 arginine residues, Arg^{637} is conserved in HGF (Arg^{647}). Arg^{639} (Lys^{649} in HGF) forms a salt bridge with RON Glu^{190} (Val^{188} in MET) and may be involved in ligand-receptor selectivity. Arg^{641} of MSPβ interacts with the backbone oxygen of RON Gly^{192} in one complex of the asymmetric unit but is disordered in the second complex, suggesting that this is not a key interaction.

RON-MSP Interaction in Solution—The AUC studies complement the crystallographic studies by investigating whether the protein partners can form complexes with a stoichiometry higher than 1:1, as observed in the crystal structure. Although crystals were only obtained with RON SPI1 and MSPβ, the receptor-ligand interactions in solution were characterized with both MSPβ and full-length MSPαβ. Analogous studies performed with MET and HGF showed a 2:2 MET SP-HGFαβ stoichiometry in solution (66). Likewise, the MET SP-HGFβ crystal structure exhibited only a 1:1 complex (58).

The SV and SE experiments revealed that single-chain MSPβ, two-chain MSPβ, MSPα, pro-MSP, MSPαβ, and RON SPI1 exist predominantly as monomers in solution (Fig. 4, A–H). The c(s) distribution profile of each protein showed a

![FIGURE 4. SV and SE analyses of MSP and RON domains. A, c(s) distribution profile of 2 μM single-chain MSPβ. B, SE profile of 14 μM single-chain MSPβ with a best fit RMSD of 0.0066 absorbance units (AU), collected at 8,000-, 12,000-, 16,000-, and 21,000-rpm rotor speeds. C, the c(s) distribution profile of 2 μM MSPα. D, SE profile of 10 μM MSPα with a best fit RMSD of 0.0102 AU, collected at 8,000, 12,000, and 18,000-rpm rotor speeds. E, c(s) distribution profiles of 5.25 μM pro-MSP and 2.1 μM MSPαβ. F, SE profile of 4 μM MSPαβ with a best fit RMSD of 0.0079 AU, collected at 6,000-, 10,000-, and 16,000-rpm rotor speeds. G, c(s) distribution profile of 1.9 μM SPI1. H, SE profile of 8 μM SPI1 with a best fit RMSD of 0.0062 AU, collected at 10,000-, 14,000-, and 20,000-rpm rotor speeds. I, c(s) distribution profiles of 1 μM MSPβ in the presence of 0.125–5 μM SPI1. The solid lines represent SV profiles of 1 μM MSPβ mixed with 0.5 μM (magenta), 2 μM (cyan), and 5 μM SPI1 (blue). The dashed lines correspond to the SV profiles of free proteins. Inset, the s_w(c) isotherm derived by integration of c(s) profiles. Fits for a 1:1 heterodimeric association were calculated with hydrodynamic constraints (s-value of 6.25 for the complex). The calculated K_d value from the nonlinear least squares analysis shown in the inset was 0.28 μM, which reflects data from two independent sets of experiments, distinguished by squares and circles. J, SE profiles of 1 μM equimolar SPI1/MSPβ mixture with a best fit RMSD of 0.0039 AU, collected at 8,000-, 12,000-, 16,000-, and 21,000-rpm rotor speeds. Solid lines, calculated global best fit distributions using an A + B ↔ AB model with mass conservation. The c(s) distributions were normalized by dividing all c(s) values by the total absorbance present in the sample. All SE profiles were globally analyzed using a single species of interaction system with mass conservation. The best fits are shown as black solid lines through the experimental data. The combined residuals in AU from the same cell at different rotor speeds are shown below the plot.](image-url)
major symmetric peak with experimental weight average sedimentation coefficient ($s_{20,w}$) that was consistent with the calculated value (Table 2) (53, 57). Moreover, the SE profiles of free RON and MSP proteins were best fitted by a monomeric species model, confirming the SV results (Table 2). With the exception of MSPβ, small amounts of higher order aggregates were detected in these samples (4–7% of the RON SPI1 at ~7.3–8.8 S, ~9% MSPα at ~6–8 S, and ~3–7% pro-MSP and MSPαβ at ~8.5–9.5 S). The amount of aggregates was independent of protein concentrations, indicating that they are probably irreversibly associated oligomers (data not shown).

MSPα exhibited a broader sedimentation boundary with an experimental $f/f_0$ of 1 (Fig. 4C), characteristic of protein heterogeneity (67). Yet a monomer model best fits the SE profiles of 10.2 S unit and a 30–44 Å increase in structural Studies of RON-MSP Complex

**Table 2**

| Proteins            | Calculated $s_{20,w}$ | Experimental $f/f_0$ | Experimental $f/f_0$ | SE MALDI |
|---------------------|-----------------------|---------------------|---------------------|----------|
| Single-chain MSPβ  | 2.96 ± 0.16           | 1.16 ± 0.30         | 1.12 ± 0.12         | 28.3 ± 29.2 |
| Two-chain MSPβ     | 2.96 ± 0.16           | 1.26 ± 0.24         | 1.13 ± 0.12         | 28.6 ± 29.2 |
| MSPα               | 4.3 ± 0.1             | 1.20 ± 0.46         | 0.99 ± 0.12         | 49.6 ± 54.0 |
| Pro-MSP            | 5.6 ± 0.1             | 1.18 ± 0.75         | 1.2 ± 0.1           | 82.1      |
| MSPαβ              | 5.6 ± 0.1             | 1.12 ± 0.56         | 1.2 ± 0.1           | 83.7 ± 82.1 |
| Sema               | 4.30 ± 0.18           | 1.21 ± 0.49         | 1.16 ± 0.12         | 51.4 ± 56.4 |
| Sema-PSI           | 4.74 ± 0.18           | 1.22 ± 0.47         | 1.2 ± 0.1           | 64.0      |
| SPI1               | 5.04 ± 0.18           | 1.31 ± 0.57         | 1.27 ± 0.12         | 78.8 ± 77.7 |
| SPI1-MSPαβ (1:1)   | 6.22 ± 0.18           | 1.28 ± 0.61         | 1.22 ± 0.12         |          |
| SPI1-MSPαβ (1:2)   | 8.1 ± 0.18            | 1.3 ± 0.79          | 1.3 ± 0.12          |          |
| SPI1-MSPαβ (2:2)   | 12.9 ± 0.18           | 1.35 ± 0.95         | 1.05 ± 1.2          |          |

a Calculated $s_{20,w}$ using HYDROPRO from structure (57).

b Calculated $s_{20,w}$ using SEDFIT with molecular weight determined by MALDI-TOF and $f/f_0$ of 1.2–1.3 (57). The estimated MALDI-TOF values for 1:1 and 2:2 SPI1-MSPαβ complexes are ~160 and 320 kDa, respectively.

* ND, not determined.

The simplest interpretation of the combined SPI1-MSPβ and SPI1-MSPαβ stoichiometry of SPI1-MSPβ was obtained by comparing the 3–6.5 S peaks based on the mass-balance conservation (Fig. 4I, inset). A nonlinear least squares analysis of $s_{20,w}$ using a heteroassociation model (A + B ↔ AB) gave an equilibrium dissociation constant ($K_D$) of ~0.28 μM with a fixed $s_{20,w}$ of 6.2 S. Analyses of SE profiles of SPI1/MSPβ mixtures confirmed the SV results (Fig. 4J), in that they were also best fit globally to the same model of a 1:1 complex with $K_D$ of ~0.15 μM. The differences in $K_D$ values derived from the SE and SV experiments are within experimental error (70).

The $c(s)$ distributions for the biologically active MSPαβ and SPI1 showed a major species at 7.7–8.15 S (Fig. 5A), consistent with the calculated $s_{20,w}$ of 8.1 S for a 1:1 SPI1-MSPαβ complex (Table 2). However, ~3–8% of the total signal in these experiments resolved as 9.5–10.5 S species (Fig. 5A), which may correspond to a higher state of receptor-ligand association. By contrast, the SPI1-MSPβ samples did not reveal any higher order species under similar protein concentrations (Fig. 4I). A complementary SE experiment of SPI1-MSPαβ association was conducted to determine the stoichiometry of this higher order protein complex (Fig. 5B-D). Initial analysis of the data (2 μM equimolar) showed a poor fit to a simple (A + B ↔ AB) model (Fig. 5E).

Therefore, the SE profiles were analyzed using two more complex models wherein the reactants reversibly associate to form a complex with either a 2:1 (2A + B ↔ AB + A ↔ A2B) or 2:2 (2A + 2B ↔ 2AB ↔ (AB)2) stoichiometry, where A corresponds to SPI1 and B corresponds to MSPαβ. These models were considered probable based on knowledge of the stoichiometry of receptor-ligand complexes involved in other signal transduction pathways. The analyses yielded much better fits with either model compared with the 1:1 association model as evidenced by the distribution of residuals (Fig. 5C–E). The dissociation constants for the 2:1 association model yielded similar dissociation constants of $K_{d1}$ ~0.2–0.3 μM and $K_{d2}$ ~0.02–0.9 μM for the 1:1 and 2:1 adducts, respectively (ranges obtained from three independent experiments). For the 2:2 SPI1-MSPαβ model, the $K_{d1}$ and $K_{d2}$ values were ~0.1–0.2 and ~2–36 μM, respectively. Both models gave $K_{d1}$ values that were consistent with the SPI1-MSPβ dissociation constant. However, in contrast to the 2:1 association, the binding affinity of the 2:2 species is at least 10-fold weaker than that of the 1:1 species, consistent with the SV experiments showing predominantly the 1:1 species and only minor higher oligomeric species. Species population analysis supports the conclusion that the 2:2 complex comprises the high oligomeric species because this model predicts that, as observed by SV (Fig. 5A), the 1:1 species predominates over the entire experimental concentration range (Fig. 5F). In contrast, the alternative 2:1 association model predicts that the populations of the 1:1 and 2:1 species change with protein concentration (Fig. 5G), which is not supported by the SV experiments.

The simplest interpretation of the combined SPI1-MSPβ and SPI1-MSPαβ ultracentrifugation experiments is that the α-chain of MSPαβ mediates the dimerization of the RON receptor. The weak binding affinity of the 2:2 SPI1-MSPαβ complex.
FIGURE 5. SV and SE analyses of MSPαβ and RON SPI1. A, c(s) distribution profiles of SPI1-MSPαβ association. Dashed lines, sedimentation profiles of free proteins; solid lines, mixtures of SPI1 and MSPαβ at different concentrations: 2 μM SPI1, 0.5 μM MSPαβ (black); 0.5 μM SPI1, 2 μM MSPαβ (red); 2 μM SPI1, 2 μM MSPαβ (green); and 4 μM SPI1, 4 μM MSPαβ (blue). B, SE profiles of a 4.5 μM SPI1 and a 3.8 μM MSPαβ mixture collected at 8,000, 12,000, and 18,000-rpm rotor speeds at 4 °C and analyzed globally using the 2:1 association model described under "Results and Discussion," with mass conservation, which yielded $K_{d1} = 0.45 \mu M$, $K_{d2} = 0.35 \mu M$, an overall reduced $\chi^2 = 0.85$, and RMSD = 0.005 AU. C, the combined residuals for a fit to a 2:1 association model. D, the combined residuals for a fit to a 2:2 association model described under "Results and Discussion," which yielded $K_{d1} = 0.16 \mu M$ and $K_{d2} = 13.8 \mu M$, $\chi^2 = 1.01$, RMSD = 0.005 AU. E, the combined residuals for a fit to a 1:1 association model described under "Results and Discussion," which yielded $K_{d1} = 0.004 \mu M$, $\chi^2 = 3.23$, RMSD = 0.009 AU. F, SPI1-MSPαβ species distributions calculated as a function of total protein concentrations using the 2:1 association model with $K_{d1} = 0.16 \mu M$ and $K_{d2} = 13.8 \mu M$. G, SPI1-MSPαβ species distributions calculated as a function of total protein concentrations using the 2:2 association model with $K_{d1} = 0.16 \mu M$ and $K_{d2} = 0.12 \mu M$. 

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complex may be physiologically relevant because of the transient nature of the signal transduction process.

Conclusion—Comparison between the crystal structures of RON SPI1-MSPαβ and MET SP-HGFβ explains the origin of receptor-ligand selectivity. Despite their identical domain architecture and their 45% amino acid sequence identity, it has been known for many years that MSP and HGF exhibit strikingly distinct binding properties to their respective receptors. Pro-MSP does not bind to RON and MSPα binds at best weakly to RON. Only activated MSP binds to RON, interactions that are mediated primarily if not solely by the β-chain. In contrast, pro-HGF and HGFα bind to MET with high affinities, although the bound pro-HGF does not activate MET (71, 72). Sequence alignment reveals that the interdomain linker regions of the α-chains of MSP and HGF vary in length and in amino acid sequences, enough to allow different orientations of the respective N domain and four Kringle domains. Thus, one would expect the spatial arrangement of the N domain and Kringle domains in pro-MSP to hinder the interaction between MSPαβ and RON Sema, hindrance that is removed upon proteolytic conversion into MSPαβ.

The solution properties of single-chain pro-MSP and MSPαβ indicated that both forms retained similar overall dimensions, suggesting that the conformational transition accompanying the MSP maturation is subtle. In contrast, the small angle x-ray scattering and electron microscopy studies of HGF revealed a compact pro-HGF molecule and an elongated, biologically active HGF revealed a compact pro-HGF molecule and an elongated, and RMSD = 0.005 AU. E, the combined residuals for a fit to a 1:1 association model described under "Results and Discussion," which yielded $K_{d1} = 0.16 \mu M$ and $K_{d2} = 13.8 \mu M$, $\chi^2 = 1.01$, RMSD = 0.005 AU. F, SPI1-MSPαβ species distributions calculated as a function of total protein concentrations using the 2:1 association model with $K_{d1} = 0.16 \mu M$ and $K_{d2} = 13.8 \mu M$. G, SPI1-MSPαβ species distributions calculated as a function of total protein concentrations using the 2:2 association model with $K_{d1} = 0.16 \mu M$ and $K_{d2} = 0.12 \mu M$.
IPT domains that promotes dimerization. Nevertheless, the best encaissement model generated for the 2:2 MET-HGFαβ involved the N and K1 domains of HGFα (66). Currently, we do not know whether MSPα alone mediates the formation of a 2:2 RON-MSPαβ complex and, if so, which of the MSPα domains are involved. At the least, functional studies of MSPα domain mutants suggest that the MSP K1-2 domains or the K3 domain alone may be involved in receptor dimerization (32). In other words, the MSP mutants lacking these domains lost cellular activities, whereas mutants missing the single N, K1, K3, or K4 domains still retained some biological activities.

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