Identification of the Residues in the Extracellular Domain of Thrombopoietin Receptor Involved in the Binding of Thrombopoietin and a Nuclear Distribution Protein (Human NUDC)*

Wei-Min Chen, Bo Yu, Qing Zhang, and Peilin Xu†

From the Key Laboratory of Gene Engineering of Education Ministry, Zhongshan University, Guangzhou 510275, China

Thrombopoietin (TPO) and its receptor (Mpl) have long been associated with megakaryocyte proliferation, differentiation, and platelet formation. However, studies have also shown that the extracellular domain of Mpl (Mpl-EC) interacts with human (h) NUDC, a protein previously characterized as a human homolog of a fungal nuclear migration protein. This study was undertaken to further delineate the putative binding domain on the Mpl receptor. Using the yeast two-hybrid system assay and co-immunoprecipitation, we identified that within the Mpl-EC domain 1 (Mpl-EC-D1), amino acids 102–251 were strongly involved in ligand binding. We subsequently expressed five subdomains within this region with T7 phage display. Enzyme-linked immunosorbent binding assays identified a short stretch of peptide located between residues 206 and 251 as the minimum binding domain for both TPO and hNUDC. A series of sequential Ala replacement mutations in the region were subsequently used to identify the specific residues most involved in ligand binding. Our results point to two hydrophobic residues, Leu228 and Leu230, as having substantial effects on hNUDC binding. For TPO binding, mutations in residues Asp235 and Leu230 had the largest effect on binding efficacy. In addition, deletion of the conservative motif WGSWS reduced binding capacity for hNUDC but not for TPO. These separate binding sites on the Mpl receptor for TPO and hNUDC raise interesting implications for the cytokine-receptor interactions.

TPO has long been studied as the primary cytokine regulator of megakaryocyte development. Its activity is mediated by the thrombopoietin receptor (Mpl), a member of the cytokine receptor superfamily (1–4). Genetic disruption of either cytokine or receptor drastically interferes with platelet production, indicating the major role TPO and Mpl play in the activation of megakaryopoiesis and platelet production (5–7). However, despite the platelet volume decrease, the megakaryocytes and platelets that are produced remain morphologically normal and are adequate to prevent severe hemophilia in murine experiments (8). This observation leaves open the possibility for other cytokines and receptors to have an overlapping biological potency. One such candidate we have investigated as an additional ligand for Mpl is known as hNUDC. Despite having no detectable sequence similarity with TPO, hNUDC has been shown to have similar megakaryopoietic effects (9, 10). TPO and hNUDC also exhibited a communal signal transduction cascade in several megakaryocytic cells expressing Mpl (11, 12).

Human NUDC was initially identified and cloned as a nuclear migration protein based on the similarity of its C terminus to that of fungal NUDC from Aspergillus nidulans (13). Numerous studies have implicated hNUDC as an endogenous factor involved in cell mitotic spindle formation, cell proliferation, cell cytokinesis, and nuclear migration in a wide variety of experimental animal cells (14–16). Although earlier studies have suggested the mechanisms by which hNUDC exerts its endogenous effects, evidence is now emerging that hNUDC also plays a critical role in human hematopoietic cells (10, 12, 17, 18). Over the past several years, progress has been made in linking the biological effects of hNUDC to Mpl (11, 12, 19).

Molecular modeling of Mpl tertiary structure shows that it is most related to the EPO receptor. Mpl is composed of a large extracellular domain (Mpl-EC), a single membrane-spanning region, and a short cytoplasmic tail (20, 21). The Mpl-EC is the principal ligand-binding region consisting of two domains, Mpl-EC-D1 and Mpl-EC-D2. Two conserved disulfide bridges are found in the N-terminal side of both domains, and WXXS motifs are located at the C-terminal regions of both Mpl-EC-D1 and Mpl-EC-D2 (22, 23). These features of Mpl-EC are also conserved in other members of the class 1 cytokine receptor family, including the granulocyte-macrophage colony-stimulating factor receptor, the granulocyte colony-stimulating factor receptor, and the interleukin family of receptors (22, 23). Despite sharing these common structural motifs, functionally significant variations are seen in the extracellular domains of cytokine receptor superfamily members. The topographical study of the structure of Mpl therefore remains of considerable value.

In this study we map the extracellular domain of Mpl using the yeast two-hybrid system and co-immunoprecipitation. Furthermore, we combine site-specific mutagenesis and phage dis-
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As a method for the comparative analysis of single amino acid variations in the Mpl-EC domain on both TPO and hNUDC binding specificities.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—The two-hybrid system and mouse monoclonal antibody against the T7 tail fiber were purchased from Clontech. Rabbit polyclonal anti-c-Mpl was purchased from Millipore (Bedford, MA). Mouse monoclonal anti-TPO was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A T7Select® cloning kit was obtained from Novagen (Madison, WI). A MutanBEST kit was obtained from TaKaRa Biotechnology (Dalian, China). The JetPEITM transfection kit was purchased from PolyPlus Transfection Co. (Illkirch, France).

Construction of the Yeast Two-hybrid Expression Vectors—Corresponding to amino acid number, different portions of Mpl were obtained via PCR with the following primers: 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 1–251; 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 1–117; 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 251–466; 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 1–251; 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 1–117; 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-CCGCGCTCGAGAGTCACAGGGAGGGACCAGG-3' for aa 102–251. EcoRI and XhoI sites were replaced by a tag complementary to the 5' end of the Mpl signal peptide sequence, and the 3' XhoI sites were replaced by a tag complementary to the 5' end of the Mpl transmembrane sequence. Each Mpl-EC sequence was mixed and annealed with both the signal peptide and the transmembrane plus intracellular sequence. This was then amplified in a fourth PCR using the primers 5'-GGTGATGGAGCAGGTGTTGGAAGCTCAG-3' and 5'-GGCCCTCTTC-3'. The final PCR products were cut with EcoRI and XhoI restriction digests and ligated into pEGFP-N1 upstream and in-frame with EGFP. The resulting expression vectors pMpl(1–466)-EGFP, pMpl(1–251)-EGFP, pMpl(251–466)-EGFP, pMpl(1–117)-EGFP, and pMpl(102–251)-EGFP were co-transfected into NIH 3T3 cells with either phNUDC-DsRed or pTPO-DsRed using JetPEITM transfection kits (PolyPlus Transfection Co.) according to the methods described previously (11).

Co-immunoprecipitation—The NIH 3T3 cells co-transfected with the Mpl-EC deletion variants and either phNUDC-DsRed or pTPO-DsRed were lysed in an IP buffer (1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA) containing protease inhibitors. A high speed centrifugation step removed cell debris from both lysates and cell culture media. Equal amounts of cell extracts and culture media (200 μl) were immunoprecipitated by addition of 5 μg of mouse monoclonal antibody raised against either hNUDC (anti-hNUDC mAb) or TPO (anti-TPO mAb) and incubated with 0.1% bovine serum albumin overnight at 4 °C. Protein A-Sepharose beads (Amersham Biosciences) were added and incubated for 2 h at 4 °C with mixing. Immune complexes were resolved by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes and Western blotted with polyclonal antibody against Mpl (anti-Mpl pAb). Horseradish peroxidase-conjugated secondary antibody and 3,3’,5,5’-tetramethylbenzidine (Amersham Biosciences) were used to develop the Western blots.

Protein Purification of hNUDC and TPO—PCR was used to amplify DNA fragments encoding the N-terminal domain of TPO (residues 1–156) using the forward and reverse primers 5'-CCGGAATTCGATGTCTCCTTGCTGGCATCA-3' and 5'-TTGGCTGCTGACTGACTCTCGTG-3'. The transmembrane domain following the intracellular sequence (residues 467–609) was amplified by PCR with specific primers 5'-TCCCTTGTGACCGCTCTGAC-TCTA-3' and 5'-TCAAAGGCTGCTGCAAATGCCTTAG-3'. The Mpl-EC regions corresponding to amino acids 1–466, 1–251, 251–466, 1–117, and 102–251 were amplified via PCR. The primers used for the amplification of these fragments are largely identical to those indicated above. However, the 5' EcoRI sites were replaced by a tag complementary to the 3' end of the Mpl signal peptide sequence, and the 3' XhoI sites were replaced by a tag complementary to the 5' end of the Mpl transmembrane sequence. Each Mpl-EC sequence was mixed and annealed with both the signal peptide and the transmembrane plus intracellular sequence. This was then amplified in a fourth PCR using the primers 5'-GGTGATGGAGCAGGTGTTGGAAGCTCAG-3' and 5'-GGCCCTCTTC-3'. The final PCR products were cut with EcoRI and XhoI restriction digests and ligated into pEGFP-N1 upstream and in-frame with EGFP. The resulting expression vectors pMpl(1–466)-EGFP, pMpl(1–251)-EGFP, pMpl(251–466)-EGFP, pMpl(1–117)-EGFP, and pMpl(102–251)-EGFP were co-transfected into NIH 3T3 cells with either phNUDC-DsRed or pTPO-DsRed using JetPEITM transfection kits (PolyPlus Transfection Co.) according to the methods described previously (11).

Generation of NIH 3T3 Cells Expressing Mpl-EC Deletion Constructs—The construction of the pMpl-EGFP, phNUDC-DsRed, and pTPO-DsRed vectors used in this study have been described previously (11). A 78-bp oligonucleotide duplex encoding an Mpl signal peptide was amplified from the pMpl-EGFP vector with the primers 5'-ATGCCCTCTG-GGCCCTCTTC-3' and 5'-TTGGCTGCTGACTGACTCTGCTG-3'. The transmembrane domain following the intracellular sequence (residues 467–609) was amplified by PCR with specific primers 5'-TCCCTTGTGACCGCTCTGAC-TCTA-3' and 5'-TCAAAGGCTGCTGCAAATGCCTTAG-3'.
sion levels of TPO fusion proteins, we routinely added a Kozak sequence GACCATGG (italic), at the beginning of the coding region. The underlined nucleotides denote the EcoRI and Xhol sites, respectively. An amplified fragment was digested with the appropriate restriction enzymes and cloned into a similarly digested pCDNA3.1 (Clontech) resulting in a plasmid designated pCDNA-TPO-His. In a similar manner, an expression construct for hNUDC was created using the plasmids pCDNA-TPO-His and pPICZaA-hNUDC-His (10) as templates. The TPO signal peptide (TS) consisting of 21 TPO amino acid residues was fused to the 5’ terminus of the hNUDC-His fusion protein in pCDNA 3.1, giving the plasmid designated pCDNA-TS-hNUDC-His.

Dihydrofolate reductase negative cells (CHO/dhfr− cells, CRL-9096, ATCC) were grown and maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 0.1 mM hypoxanthine, 0.016 mM thymidine, 100 mM methotrexate, and 10% dialyzed fetal bovine serum. All cell cultures in this study were performed at 37 °C in a 5% CO2 humidified atmosphere. CHO/dhfr− cells were co-transfected with 5 µg of pSV2-dhfr, a plasmid expressing the dihydrofolate reductase gene, with either pcDNA3.1-TPO-His (5 µg) or pcDNA3.1-TS-hNUDC-His using CaCl2 transfection kits (Beyotime, China) according to the manufacturer’s protocol. Following transfection, CHO/dhfr− cells were cultured in non-selective medium for 3 days. Stably transfected clones expressing either TPO or hNUDC were selected for their ability to grow in 10% dialyzed fetal bovine serum, 500 µg/ml G418 Iscove’s modified Dulbecco’s medium lacking hypoxanthine and thymidine. After 2 weeks, hygromycin-resistant cells were subjected to increasing levels of methotrexate at concentrations of 0.2, 0.8, 1.0, 4.0, and 10 µM. The supernatant from methotrexate-resistant cell cultures were confirmed by Western blot assay.

The medium containing the expression proteins were subjected to nickel affinity chromatography (Clontech) to purify hNUDC and TPO and subsequently washed with 3 column volumes of 300 mM NaCl, 20 mM sodium phosphate, pH 8.0. The protein was eluted with the same buffer containing 300 mM imidazole.

**Construction of the Phage Display Mpl Subdomains**—Five subdomains corresponding to different portions of Mpl-EC-D1 were amplified by PCR using the following primers: 5′-CCGGGAATTTCCGAGCAGGCTAGTGACGG-3′ and CCGAACTTTGTCAGCTGATGACCTG-3′ for aa 102–164; 5′-CCGGGAATTTCCGAGCCCTGTGATGACCTG-3′ and 5′-CCCAAGCTTAGTCAAGGAGGGAGCCAG-3′ for aa 165–251; 5′-CCGGGAATTTCCGAGCCCTGTGACGGATCTCAG-3′ and 5′-CCCAAGCTTAGTCAAGGAGGGAGCCAG-3′ for aa 206–251. The DNA fragments were digested with EcoRI and HindIII (underlined) and ligated into a pTZ57R/T vector (Fermentas, Burlington, Canada). DNA inserts were confirmed by DNA sequence analysis. Depending on the size of the aa residue, the inserts were cloned into the corresponding sites of the T7Select 415-1b (<50 aa) or T7Select10-3b (≥50 aa) phage arms to create the constructs pT7Select10-Mpl(102–164), pT7Select10-Mpl(165–251), pT7Select415-Mpl(165–205), pT7Select415-Mpl(180–229), and pT7Select415-Mpl(206–251). Phage packaging, amplification, and titration were performed according to the manufacturer’s instructions.

**Alanine Scanning Mutagenesis**—Point mutations in the ligand-binding sites on Mpl-EC-D1 were generated by standard PCR-based site-directed mutagenesis using a TaKaRa MutanBEST kit according to the manufacturer’s instructions. The cDNA encoding for aa 206–251 of Mpl-EC-D1 (wild type) was subcloned into pTZ57R/T vector to obtain a pT757R/T-Mpl (206–251) plasmid suitable for the mutagenesis reactions. Either single or double residues were converted to Ala using PCR with pTZ57R/T-Mpl (206–251) as a template. The mutagenic primers are summarized in Table 1. Once the mutations were confirmed by DNA sequence, the inserts were extracted by E.Z.N.A.gel extraction kit (Omega Bio-Tek, Guangzhou, China). Subsequently, they were all subcloned in T7Select 415-1b vector to allow comparison with phage display expression between mutant and wild-type receptors. Each phage clone was further verified by PCR with primers flanking the insertion site and sequencing of the insert.

**Binding of Phages to Immobilized hNUDC or TPO**—Protein-charged ELISA plates were coated with 200 µg hNUDC-His or TPO-His overnight at 4 °C. Plates were blocked by the addition of phosphate-buffered saline with 5% nonfat milk for 1 h at room temperature. After five washes with phosphate-buffered saline, phage samples (1 × 1010 pfu/well) in phosphate-buffered saline buffer were added to hNUDC-His- or TPO-His-coated wells and incubated for an additional 1 h at 37 °C. Following incubation, unbound phages were removed by washing the wells three times with phosphate-buffered saline containing 0.05% Tween 20, and the amount of retained phages was determined with phage-specific antibodies, anti-T7 tail fiber monoclonal antibody (anti-T7 fiber mAb) diluted 1:2000, followed by 1 h of incubation with a 1:1000 dilution of horseradish peroxidase-labeled anti-mouse secondary antibody. Development of the plates was done by adding 3,3′,5,5′-tetramethylbenzidine, and the color change was quantified at 450 nm on a microplate spectrophotometer. Null phage, which did not carry any insert, was used as background control.

**Competitive Inhibition ELISA**—Phage expressing the Mpl-ligand-binding domain and its derivative mutants (1 × 109 pfu/well) were mixed with serial dilutions of increasing TPO-His concentrations ranging from 0 to 500 nM. These phage mixtures were then added to the hNUDC-His-coated microwells. Following incubation, microtiter plates were washed thoroughly, and bound phage were detected with anti-T7 tail fiber mAb as described above for phage ELISA. IC50 values were calculated using the Statistical Package for the Social Sciences software (SPSS Statistics 17).

**Molecular Modeling**—A model of Mpl-EC-D1 was constructed using the crystal structure of the extracellular domain of EPO receptor (EPO-binding protein (EBP)) as a template (PDB accession number 1ERN). The sequences of Mpl-EC-D1 (NCBI Reference Sequence accession number NP-005364) and
EBP (GenBank™ accession number AAA52403) were aligned based on conserved amino acids, including the Cys-Cys disulfide bonds and the WXXWS boxes according to the guidelines described by Deane et al. (23) for class 1 cytokine receptors. The Mpl-EC-D1 molecular model, both A–G and A’–G’ β-strains, was initially developed according to the corresponding domain of EBP using the Swiss-Model server (24). The molecular complex of Mpl-EC-D1 and TPO (PDB accession number 1V7M) was generated by submitting the relevant protein structures to the program Cluspro 2.0 (25). The ligand-receptor complex was manually assembled into the most complementary orientation using PyMOL version 0.99 (DeLano Scientific).

**TABLE 1**

Mutagenesis of residues in the Mpl binding domain by phage display

The effect on the binding of TPO or hNUDC by phage displayed mutant peptides as assayed using ELISA. Results shown represent the mean ± S.D., n = 6 replicates.

| Mutagenesis primers | Mutant positions | Binding percentage to the wild-type |
|---------------------|------------------|-------------------------------------|
| Wild-type           |                  |                                     |
| F:GCATCTGGCTGCACTGGC | 225 A             | 91 ± 02 98 ± 04 |
| R:GTTGCCAGGCCATGCTGCA  |                  |                                     |
| F:GCATGGCTGCACTGGCAGC | 226 A             | 93 ± 03 92 ± 01 |
| R:GGAATTGGCCAACTGGCTGGA  |                  |                                     |
| F:GCAGTGCAGCTGGCAGCAGA | 227 A             | 99 ± 01 97 ± 04 |
| R:GTAGGATTGGCCAAGCTGGAG  |                  |                                     |
| F:GCAGCTGGCAAGGAGAACTG | 228 A             | 57 ± 09 98 ± 04 |
| R:CTGAAAGGTAGAGTTGGCC  |                  |                                     |
| F:GCAGCTGGCAAGGAGACCTG | 229 A             | 94 ± 05 97 ± 07 |
| R:TAGGCAGTAAAGATGGCTGGA  |                  |                                     |
| F:GCAGCACGGACACTGATGAGG | 230 A             | 63 ± 03 95 ± 03 |
| R:CTGAAAGGTAGAGTTGGCC  |                  |                                     |
| F:GCAAAGCAAGCATGTAGAGATC | 231 A             | 95 ± 02 94 ± 06 |
| R:TAGGCAGTAAAGATGGCTGGA  |                  |                                     |
| F:GCAGAACCTGATGGATCTC | 232 A             | 95 ± 07 94 ± 06 |
| R:GCGAACAGCTGACGCTAGA  |                  |                                     |
| F:GCAGCTGATGTGGATCTCCCTC | 233 A             | 99 ± 06 98 ± 03 |
| R:GCTGCGAGCTGACGCTAGA  |                  |                                     |
| F:GCAGATGGATCCTGCTGGT | 234 A             | 91 ± 24 90 ± 05 |
| R:TTGCTGCGACGTGACAGCA  |                  |                                     |
| F:GAGGAGATCTGCTGGTGGG | 235 A             | 88 ± 07 64 ± 07 |
| R:AGTTGTGGTGGCTGAGCTG  |                  |                                     |
| F:GACATGTCGCTCTGCTGGT | 236 A             | 97 ± 04 95 ± 08 |
| R:ATCAAGTGGGTGGTGGCTG  |                  |                                     |
| F:GACTCTCTGCTGCTGGTGGT | 237 A             | 90 ± 21 95 ± 10 |
| R:CCATCTAGGTGTCTGAGCTG  |                  |                                     |
| F:GACCTGCTGCTGGTGGGGA | 238 A             | 96 ± 04 92 ± 08 |
| R:GATCCATGAGCTGTGGCTG  |                  |                                     |
| F:GACAGTGGCTGCTGGGATCC | 239 A             | 91 ± 07 43 ± 06 |
| R:GGAATACCCTATACAGTGTG  |                  |                                     |
| F:GCAGGAGCAGCTGGAGAAGCT | 228 230 A–A       | 18 ± 03 80 ± 05 |
| R:CTGACAGCTGAGATAGTGCC  |                  |                                     |
| F:GGAAGATGCTGCTGAGTGGC | 235 239 A–A       | 92 ± 09 09 ± 04 |
| R:GGAAGATAGCTGAGTGTGTCT |                  |                                     |

*Positions for mutagenesis in pT7Select-Mpl(206–251) are underlined.
*b The WGSWS residues are deleted in pT7Select-Mpl(206–251).
RESULTS

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Characterization of the Binding Domains of Mpl with TPO or hNUDC in Yeast Two-hybrid System—The first step toward mapping the Mpl-EC binding regions for both TPO and hNUDC was to examine the protein-protein interactions in a yeast two-hybrid system. A series of Mpl-EC deletion constructs were created in pGBK7T-BD (Fig. 1A). TPO and hNUDC constructs were created in pGADT7-AD. When co-expressed, the presence of interactions between the receptor and cytokine constructs will result in the transcription of reporter genes that code for the amino acid nutritional markers adenine and histidine. Two deletions, Mpl(1–117) and Mpl(251–466), failed to induce yeast cell growth. The other deletions Mpl(1–466), Mpl(1–251), and Mpl(102–251) induced growth of the yeast cells in a manner comparable with the positive control (Fig. 1B, left panel). These results were corroborated by the galactosidase assays. As indicated by the blue coloration, galactosidase activity in yeast cells with the Mpl(1–466), Mpl(1–251), and Mpl(102–251) constructs was comparable with the positive control (Fig. 1B, right panel). In contrast, yeast containing the Mpl(1–117) and Mpl(251–466) constructs showed no galactosidase activity. These results indicate that the Mpl-EC region encompassing aa 102–251 is sufficient for some level of interaction with hNUDC and TPO.

Specific Co-immunoprecipitation of Mpl with TPO or hNUDC in Mammalian Cell—Co-immunoprecipitation experiments were performed in the NIH 3T3 cell line to investigate the specific binding interactions between Mpl-EC deletions with TPO or hNUDC. In an effort to facilitate cytokine secretion, all of the deletion constructs of Mpl were designed to contain a signal peptide, the transmembrane domain and the intracellular domain (Fig. 2A). These cDNA fragments were subcloned in the mammalian expression vector pEGFP-N1 upstream and in-frame with EGFP coding sequences. NIH 3T3 cells were stably transfected with these plasmids, and the subsequent cell lysates were Western blotted with anti-Mpl pAb (Fig. 2B). The molecular masses of the receptor portions were in the range of 111 kDa for Mpl(1–466)-EGFP, 84 kDa for Mpl(1–251)-EGFP, 85 kDa for Mpl(251–466)-EGFP, 71 kDa for Mpl(1–117)-E-GFP, and 66 kDa for Mpl(102–251)-EGFP. These values were all in line with calcu-
lated molecular weights (Fig. 2B). The NIH 3T3 cells stably expressing Mpl-EC deletion constructs were co-transfected with expression plasmids encoding either hNUDC or TPO for a period of 48 h. As reported previously, hNUDC is secreted outside the cell when co-expressed with Mpl. Similarly, TPO is released into the culture medium as a composite cytokine. The mixtures of cell lysates and culture medium were subjected to, depending on the cytokine, immunoprecipitation with anti-hNUDC or anti-TPO mAb. The strongest interactions were observed with Mpl(1–466)-EGFP, Mpl(1–251)-EGFP, and Mpl(102–251)-EGFP co-precipitated hNUDC as detected by anti-Mpl pAb Western blot (Fig. 2C). Similar results were observed when co-expressing TPO together with Mpl-EC deletions (Fig. 2D). In contrast, co-expression of Mpl(1–117)-EGFP or Mpl(251–466)-EGFP with either TPO or hNUDC did not result in interactions. These results reinforce that the region encompassing aa 102–251 within the Mpl-EC-D1 is sufficient for interactions with both hNUDC and TPO.

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**Co-immunoprecipitation of Mpl deletions with TPO or hNUDC in NIH 3T3.**

**A.** Schematic representation of the EGFP-tagged Mpl deletion constructs tested in the co-immunoprecipitation assay. Numbers refer to amino acid positions that have been cloned into pEFGP-N1. The signal peptide, transmembrane, intracellular domains, and green fluorescent protein are abbreviated as SP, TM, IC, and EGFP respectively. **B.** Western blot (WB) analysis of total lysates. An equal amount of proteins (5 μg) from extracts of NIH 3T3 cell stably transfected with Mpl deletion constructs were loaded onto a 12% SDS-PAGE. Western blot analysis of expressed Mpl deletions was performed with anti-Mpl polyclonal antibody (pAb). C. Co-immunoprecipitate of Mpl deletions with hNUDC. NIH 3T3 cells stably transfected with different EGFP-tagged Mpl deletions were co-transfected with phNUDC-DsRed. Culture media and cell lysates were immunoprecipitated (IP) with the anti-hNUDC mAb. Western blot analysis of the immunoprecipitates was performed with anti-Mpl polyclonal antibody. **D.** Co-immunoprecipitate of Mpl deletions with TPO. NIH 3T3 cells stably transfected with different EGFP-tagged Mpl deletions were co-transfected with pTPO-DsRed. Culture media and cell lysates were immunoprecipitated with the anti-TPO mAb. Western blot analysis of the immunoprecipitates was performed with anti-Mpl pAb.
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To evaluate cytokine binding receptor in a more quantitative way, the recombinant phages displaying the different regions of Mpl-EC-D1 to either hNUDC or TPO were evaluated by phage titrations. Within the range of phage titers from $1 \times 10^7$ to $1 \times 10^{10}$ pfu/well, binding of T7Select10-Mpl(165–251) and T7Select415-Mpl(206–251) phages to hNUDC or TPO increased in a concentration-dependent manner (Fig. 4B). However, T7Select415-Mpl(165–205), T7Select415-Mpl(102–164), and T7Select415-Mpl(180–229) phages displayed no binding affinity for hNUDC or TPO regardless of concentration (Fig. 4B). Notably, ligand binding affinity for T7Select10-Mpl(165–251) was somewhat lower than that for T7Select415-Mpl(206–251). This result may perhaps be explained by lower DNA copy numbers per phage in a T7Select10-3b system compared with that of T7Select415-1.

**Mutagenesis of Mpl-binding Sequence in Phage Display**—To characterize the residues critical to the binding interaction, we carried out Ala scanning within the 206–251 region of Mpl-EC-D1. Site-directed mutagenesis was used to generate sequential substitutions of each amino acid for Ala in T7Select415 phage constructs. Each of the resulting mutant peptides was then tested for its ability to bind either hNUDC or TPO relative to wild-type pT7Select415-Mpl(206–251). As shown in Table 1, the majority of mutant variants displayed a weak reduction in binding affinity (<10%) when compared with the wild type. In contrast, the L228A and L230A mutants resulted in a more marked reduction in the hNUDC bound. Compared with the wild type, there was a decrease of 37 and 43%, respectively (Table 1). Two other Ala mutations D235A and L239A revealed decreases in TPO binding capability of 36 and 57%, respectively (Table 1).

To investigate if double substitutions lead to a further decrease of ligand binding, we generated the following two sets of mutations: L228A/L230A and D235A/L239A. Assay revealed that the binding ability of the double mutants was essentially compromised. In comparison with wild type, the level of hNUDC bound to the L228A/L230A mutant was only 18% of wild type, and the level of TPO bound to the D235A/L239A mutant was only 9% of wild type (Table 1). We also investigated the influence the conserved WGSWS motif would have on hNUDC or TPO binding. An additional deletion experiment was conducted by removal of the WGSWS amino acids in the 206–251 region of Mpl-EC-D1. As illustrated in Table 1, this motif has no apparent effect on TPO binding but reduced the level of hNUDC binding by about 19% compared with wild type.

**Homology Modeling**—An Mpl-EC-D1 model was built based on the alignment of its sequence with that of EBP, which has a resolved crystallographic structure (26). The overall amino acid alignment revealed a low identity score between Mpl-EC-D1 and EBP (28%). Nevertheless, the general location of certain structurally important residues is conserved between Mpl-EC-D1 and EBP protein structures. These include the conservation of two Cys pairs, several hydrophobic and polar residues, as well as the WXXWS motif (Fig. 5A). Based on the predicted model, Mpl-EC-D1 is made up of two fibronectin type III

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**Figure 3.** Purification of recombinant fusion proteins. A, expression of TPO-His. Lane M, molecular mass protein markers; lane 1, culture supernatant from CHO/dhfr expressing TPO-His; lane 2, fraction containing purified TPO-His eluted from nickel-charged affinity chromatography; lane 3, Western blot analysis to detect expression of TPO-His with an anti-His mAb. B, expression of hNUDC-His. Lane M, molecular mass protein markers; lane 1, culture supernatant from CHO/dhfr expressing hNUDC-His; lane 2, fraction containing purified hNUDC-His eluted from nickel-charged affinity chromatography; lane 3, Western blot analysis to detect expression of hNUDC-His with an anti-His mAb. The arrows indicate the positions of the TPO-His and hNUDC-His fusion proteins.
(FnIII)-like domains. Each contains seven antiparallel β-strands, denoted as A–G and A’–G’, respectively (Fig. 5, A and B). It is also characterized by a large insert sequence (Ala172–Gly213) located with the second FnIII-like domain flanked by the D and F β-strands (Fig. 5, A and B). This insertion was not homologous to any other known structure associated with cytokine receptors and therefore was considered to most likely be unrelated to ligand binding and functioning (23). The WGSWS sequence located in the C-terminal region of the second FnIII-like domain of Mpl runs roughly antiparallel to the F’ β-strand (Fig. 5B). Furthermore, the two disulfide bridges between the four conserved Cys in the crystal structure are present in our model. The residues associated with hNUDC binding, Leu228 and Leu230, are located in the F’ β-strand. The Asp235 and Leu239 residues involved in TPO interactions are situated in the loop region between the adjacent F and G’ β-strands (Fig. 5B). In comparison, the critical determinants of EPO binding, residues Phe93, Met150, and Phe205, are located at the E–F, B’–C, and F’–G’ loops of EBP, respectively (27, 28). Interestingly, the Phe205 residue located in the F’–G’ loop of EBP is situated in the analogous position to Leu239 in Mpl (Fig. 5B).
Homology Modeling of Deletion Variants—The Swiss-Model server was used to construct models to allow us to further rationalize the tertiary structures of the residues 102–164, 165–251, 165–205, 205–251, and 180–229 from Mpl-EC-D1. Our phage display binding analysis revealed the residues 206–251 to be most involved with affinity binding to hNUDC and TPO (Fig. 4). When this smaller stretch of amino acids was modeled individually, it was shown that the partial construct closely matched the model in Mpl-EC-D1. This suggests a similar domain arrangement in the Mpl-EC-D1 and in the deletion. The best superimpositions include E'/H11032, F'/H11032, and F'/H11032–G'/H11032–strands together with the turn linking loops of E'/H11032–F'/H11032 and F'/H11032–G'/H11032 (Fig. 6A). A structural similarity search revealed a model of the deletion domain 102–164 lacking C-terminal residues 151–164 topologically similar to that of Mpl-EC-D1 except for shifts in turn of the A'/B'–B'/C' loops (Fig. 6B). Unfortunately, the software was unable to model the 165–205, 180–229, and 165–251 subdomain structures because of the poor identity of the primary sequences. According to our model, these three deletion constructs all contain residues that are extraneous when compared with other comparable cytokine receptors (23). Although it might be possible that such insertions would disrupt the predicted structural conformations, it should be noted that residues 165–205 are unlikely to induce profound structural alterations, because this deletion displays a binding ability with both TPO and hNUDC in phage display (Fig. 4).

Predicting the Mpl-EC-D1 and TPO Complex—In view of the current lack of x-ray crystallographic or NMR structural information on hNUDC, it is difficult to predict how exactly this ligand interacts with Mpl. We are currently carrying out mutagenic and structural analyses of hNUDC in our laboratory. Homology modeling and deeper experimental exploration of the hNUDC to Mpl complex will occur in the near future. Nonetheless, information from the above studies together with the site-directed mutagenesis of TPO (29, 30) allowed us to model Mpl-TPO interactions. Specifically, we used molecular modeling programs to generate the homodimer Mpl complexed to TPO using the structure of the EPO-EBP complex as a template (PDB accession number 1CN4). The crystal structure of TPO
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We have previously reported on the overlapping biological function of hNUDC and TPO as mediated through the receptor Mpl (9–12, 19). This drove our direction of inquiry to investigate the binding mechanism associated with the cytokine-receptor interactions. In this study, our approach was to use a stepwise application of different molecular techniques to determine the minimal binding domain within Mpl for these ligands. This would allow us to characterize and compare the binding sites for hNUDC and TPO in the Mpl-EC.

Our yeast two-hybrid and co-immunoprecipitation results demonstrated that the amino acid residues 102–251 located in the C terminus of Mpl-EC-D1 are sufficient for affinity binding of both hNUDC and TPO (Figs. 1 and 2). This result is in keeping with the findings of several other independent approaches in the literature (33–35). It is also consistent with the fact that the Mpl-EC-D2 is not involved in ligand binding but is instead needed for receptor activation (36). With what we believe to be a novel use of a T7 phage display system, we were able to further refine our knowledge of the Mpl-EC-D1 and characterize the 45-amino acid long peptide necessary for both hNUDC and TPO binding (Fig. 4).
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Subsequently, we subjected the Mpl-EC-D1 to Ala scanning mutagenesis in an effort to identify the amino acid residues most involved with binding. The two residues Leu\textsuperscript{228} and Leu\textsuperscript{230} in the putative binding domain of Mpl-EC-D1 were found to most affect hNUDC binding. In addition a double mutant at these two sites resulted in a further decrease in binding affinity. TPO binding was most affected by the mutation of residues D235A and L239A. Likewise, a double mutation at these sites resulted in a synergistic decrease in TPO binding (Table 1).

We were also able to demonstrate that TPO can compete with hNUDC for binding to the wild-type pT7Select\textsuperscript{415-Mpl(206\textendash251)} phage in a concentration-dependent manner (Fig. 8, A and B). This suggests that occupancy at one ligand site may either occlude the other binding site or alter it in an allosteric manner. Furthermore, our competition assay of four mutated residues supports the conclusion that hNUDC and Mpl interact stably. In addition a double mutant at these two sites resulted in a further decrease in binding affinity. TPO binding was most affected by the mutation of residues D235A and L239A. Likewise, a double mutation at these sites resulted in a synergistic decrease in TPO binding (Table 1).

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Glu277 and Glu278 thought to be involved in the interleukin-6 binding loop of interleukin-6 receptor are also found in the F′–G′ region (40). An alignment of the F′–G′ domain of the several class 1 cytokine receptors highlights a couple of common features (Fig. 9). As with all class 1 cytokine receptors, the WXWX motif is present. In addition, the positions at which the hydrophobic residues Leu228 and Leu239 lie on hNUDC are found to be conserved as hydrophobic in the other cytokine receptors. This finding suggests that the mechanism in which hNUDC binds to Mpl may have correlates in all class 1 cytokine receptors.

The combination of site-directed mutagenesis and molecular modeling has led to a first pass characterization of the Mpl-TPO ternary complex formation. The interaction of Mpl-EC-D1 with TPO is likely very similar to the interaction between EPO and EBP (32). In the Mpl-EC-D1, Leu239 in the F′–G′ loop coincided with Phe205 of EBP to generate the binding surface with Phe214 of TPO through a hydrophobic interaction on site 1. The side chain of Asp235 donated by the F′–G′ loop of Mpl-EC-D1 forms salt bridges with the side chains of Arg140 and Lys14 of TPO on both sites 1 and 2 (Fig. 7). This residue may play a key role in the receptor dimerization or in stability of the TPO-Mpl complex. However, confirmation of this speculation will require more extensive experimental investigation.

An unexpected difference between the Mpl-EC-D1 and EBP was the disparate role the E–F and B′–C′ loops play ligand binding. In EBP, Phe92 and Met303 located in the E–F and in B′–C′ loops, respectively, are found to be important for EPO binding (27, 28). Our deletion mutants containing either the E–F or B′–C loop regions within the Mpl-EC-D1 did not show any interaction with TPO (Figs. 1 and 2). The simplest explanations would be that the residues located in E–F and B′–C′ loops would be involved in low affinity binding or involved in an obligatory intermediate step for maintaining the correct orientation of the ligand-receptor complexes. However, the scanning deletion technique is more structurally disruptive than scanning point mutagenesis. At present, it cannot be excluded that the presence of other key residues, even in the Mpl-EC-D1, may play a key role in the receptor cooperation or in stability of the TPO-Mpl complex. However, confirmation of this speculation will require more extensive experimental investigation.

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In summary, the experiments revealed that cytokine binding to the Mpl-EC-D1 involves amino acid residues 206–251. Mutational analysis of individual candidate amino acids and competition assays suggest several candidate residues in the F′–G′ domain as potential sites of interaction with hNUDC or
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TPO. Additionally the WGSWS motif was determined to be important for hNUDC binding. This is an important step in understanding the relationship between receptor structure and the mechanisms behind receptor activation. Further functional analysis will be required to completely map the interaction of Mpl with its ligands using cell-based bioassays.

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