Communication

Synthesis and Characterization of Insulin-like Growth Factor-binding Protein (IGFBP)-7

RECOMBINANT HUMAN mac25 PROTEIN SPECIFICALLY BINDS IGF-I AND -IIa

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Youngman Oh, Srinivasa R. Nagalla, Yoshitaka Yamakawa, Ho-Seong Kim, Elizabeth Wilson, and Ron G. Rosenfeld
From the Department of Pediatrics, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201

The mac25 cDNA was originally cloned from leptomeningeal cells and subsequently reisolated through differential display as a sequence preferentially expressed in senescent human mammary epithelial cells. The deduced amino acid sequence of the human mac25 propeptide shares a 20–25% identity to human insulin-like growth factor-binding proteins (IGFBPs), suggesting that mac25 could be another member of the IGFBP family.

In the present study, we have generated recombinant human mac25 (rh-mac25) in a baculovirus expression system and assessed its affinity for IGFs and have evaluated the pattern of expression of the mac25 gene in human tissues. Binding of 125I-IGF-I and 125I-IGF-II to rh-mac25 was demonstrated by Western ligand blotting after non-denaturing polyacrylamide gel electrophoresis and by affinity cross-linking with as little as 2 nM rh-mac25. Specificity of rh-mac25 binding to 125I-IGFs was demonstrated by competition for rh-mac25 binding with unlabeled IGFs, but not with [QAYLL]IGF-II analog, which has 100-fold less affinity for IGFBPs. In comparison with IGFBP-3, rh-mac25 has at least a 5–6-fold lower affinity for IGF-I and 20–25-fold lower affinity for IGF-II. mac25 mRNA was detectable in a wide range of normal human tissues, with decreased expression in breast, prostate, colon, and lung cancer cell lines.

In conclusion, mac25 specifically binds IGFs and constitutes a new member of the IGFBP family, IGFBP-7. Its wider distribution in normal tissue and lower expression in several cancer cells indicate that IGFBP-7 may function as a growth-suppressing factor, as well as an IGF-binding protein.

It consists of six distinct proteins, classified as IGFBP-1 to -6, which display no sequence homology to the IGF receptors (1–3). IGFBPs bind IGF peptides with high affinity and regulate the biological activities of the IGFs (4–12). Amino acid sequence analysis has revealed that human IGFBPs show 50–60% similarity and contain 16–18 conserved cysteines at the NH2- and COOH-terminal regions (1).

The mac25 gene, residing on chromosome 4q12 and encoding a pre-protein of 277 amino acids, has been cloned and sequenced in leptomeningeal and mammary epithelial cells, compared with their counterpart tumors, suggesting that mac25 may play a role in growth-regulatory pathways that are abrogated in meningiomas and breast carcinoma (13, 14).

Although the mature protein has yet to be identified, the deduced amino acid sequence of the mac25 pre-propeptide shows an overall 40–45% similarity and 20–25% identity to IGFBPs. Furthermore, mac25 contains the common IGFBP motif (GGCCXXCX) at the NH2 terminus, in a region containing a cluster of 12 conserved cysteines, of which 11 are found in mac25, suggesting that mac25 is another member of the IGFBP family.

In this study, we describe the successful expression of recombinant human mac25, using a baculovirus system, and demonstrate that the 27-kDa mac25 protein specifically binds IGFs, thereby meeting criteria necessary for renaming it IGFBP-7.

EXPERIMENTAL PROCEDURES

Peptides and Proteins—Recombinant human IGF-I was purchased from Bachem (Torrance, CA), and recombinant human IGF-II was provided by Eli Lilly (Indianapolis, IN). Recombinant human IGFBP-3a, a non-glycosylated 29-kDa core protein which was expressed in E. coli, was a generous gift from Celtrix (Santa Clara, CA). Highly specific rabbit polyclonal antibody, nIGFBP-3g1, was raised in our laboratory against glycosylated IGFBP-3 (9). [Gln6,Ala7,Tyr18,Leu19,Leu27]IGF-II ([QAYLL]IGF-II), a synthetic IGF-II analog, was synthesized as described previously (15). Iodination was performed by a modification of the chloramine-T technique, to a specific activity of 350–500 Ci/µg for IGF-I and IGF-II and 100 µCi/µg for IGFBP-3c (c-c)peptides (10). Human multiple tissue Northern blots were purchased from Clontech (Palo Alto, CA).

Cloning and Expression of Recombinant mac25 Protein—Initially, a partial cDNA fragment was generated by reverse transcription-polymerase chain reaction, using RNA isolated from the human breast cancer cell line Hs578T as described previously (16). The primers used for amplification were 5′-primer TGCGAGCAAGGTCTTCAAC and 3′-primer CACCCGGAGGATCTGTC (corresponding to nucleotides 479–498 and 628–647 of L198182, GenBankTM sequence). The partial cDNA was subcloned into pGEM-T vector (Promega, Madison, WI) and confirmed by sequencing. An expression cDNA library prepared in ZapExpress (Stratagene, La Jolla, CA) with Hs578T mRNA was screened with the partial cDNA fragment, as described previously (16), and full-length clones were isolated. Full-length sequence was identical to the published mac25 sequence (13).

To express recombinant protein, a FLAG epitope sequence (DYKD-DDDK) was added at the COOH terminus by use of polymerase chain reaction. Primers (A) 5′-GCCCATTTCAAGCCCGGTTTG (661–682) and (B) 5′-GCGCCCTCGACGACTTCTACCTGTCAGTGGTGGCCTTTTTTCTGCTAT (827–844), which was designed with the FLAG sequence followed by a stop codon and a restriction site for XhoI, were used, and the resulting polymerase chain reaction product was digested with SmaI and XhoI restriction enzymes and ligated into full-length cDNA digested with the above enzymes, to replace the COOH terminus. After sequencing, the FLAG-tagged mac25 cDNA was subcloned into baculovirus expression vector pFASTBAC1 (Life Technologies, Inc.).

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mac25-pFASTBAC1 construct was transfected into Sf9 insect cells and positive viral recombinants were isolated, using the vendor's protocols. Western immunoblots were performed with the FLAG sequence specific anti-M2 antibody (Eastman Kodak).

**Protein Purification and Sequencing**—Large scale protein purification was begun by infecting 10^6 HI-5 insect cells at a multiplicity of infection of 3 at 27 °C for 3 days. The media from the infected cells were collected and concentrated, and the resultants were bound to an anti-M2 antibody affinity column overnight at 4 °C. The column was washed three times with 5 ml of HBS (20 mM Hepes, pH 7.8, 150 mM NaCl), and the protein was eluted with four 1-ml washes with HBS containing FLAG peptide (lanes 3–6), or the purified rh-mac25 after pooling and concentration (lane 7). Silver staining of the purified rh-mac25 and nonglycosylated IGFBP-3 was performed as described above.

**RESULTS**

**Construction and Expression of FLAG Epitope-tagged mac25**—Fig. 1A is an immunoblot of the fractions collected during purification of FLAG-tagged mac25, and the purified concentrated protein on nonreducing 12% SDS-PAGE. The major antibody-specific protein is 27 kDa, with a minor higher molecular weight antibody-specific protein of ~54 kDa (presumably dimers of the mac25 protein). Analysis of the purified recombinant human (rh)-mac25 protein on a nonreducing 12% SDS-PAGE and subsequent silver staining (Fig. 1B), show a protein of approximately 99% purity and a molecular weight of 27,000. The N-terminal sequence of the purified rh-mac25 was SSSDTGCPE, indicating that human mac25 contains 26 residues of signal peptide sequence and 251 amino acids of the mature protein, which is 18–22% identical to other mature IGFBPs (most identical to IGFBP-3).

Since the deduced amino acid sequence analysis revealed that mac25 contains one potential N-glycosylation site located at amino acid 171 (Asn-Val-Thr), we treated mac25 with various concentrations of Endo F (120–480 milliunits) to cleave the N-glycosylated carbohydrates. As shown in Fig. 2A, the size of mac25 was reduced to approximately 25 kDa following treatment with 480 milliunits of Endo-F, indicating that the secreted rh-mac25 is a glycosylated protein with 2 kDa of N-linked sugars and a 25-kDa core. Parallel treatment of human serum IGFBP-3 with Endo F reduced the 41- and 39-kDa glycosylated species to a 29-kDa core protein (Fig. 2B).

**Characterization of mac25 as IGFBP-7**—To test whether mac25 is capable of binding to IGF peptides, we performed WLB and IGF affinity cross-linking. As shown in Fig. 3A, when we employed WLB under denaturing conditions with 125I-IGF-I (upper panel) or 125I-IGF-II (lower panel), rh-mac25 was not detected, at concentrations of rh-mac25 ranging from 1.5 to 150 nM, although 5 nM rhIGFBP-3 was detected as a 29-kDa band with either 125I-IGF-I or 125I-IGF-II. However, when rh-mac25 was electrophoresed by non-denaturing PAGE, rh-mac25 was faintly detected at a concentration of 30 nM and clearly

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1. Purification of FLAG epitope-tagged mac25.** A, purification and Western immunoblotting of baculovirus recombinant mac25/FLAG using anti-M2 monoclonal antibody. Lanes represent 10^6 HI-5 infected cell protein of approximately 99% purity and a molecular weight of 27,000. The NH2-terminal sequence of the purified rh-mac25 (10 μg) was determined (Biotechnology Laboratory in the Institute of Molecular Biology, University of Oregon) by Edman degradation using a 470A gas-phase protein Sequencer equipped with a 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA). B, silver staining of the purified rh-mac25 after washing at high stringency as described previously (16).

![Fig. 2](https://example.com/fig2.png)  
**Fig. 2. Deglycosylation of rh-mac25 and normal human serum IGFBPs with Endo F treatment.** Western immunoblot of rh-mac25 with anti-M2 monoclonal antibody (A) and Western ligand blot of normal human serum IGFBPs with 125I-IGF-I (B) after treatment with various concentrations of Endo F at 37 °C for 3 h. Lanes represent 40 ng of rh-mac25 alone or 2 μl of human serum alone (lane 1) or after Endo F treatment (120 milliunits of Endo-F, lane 2; 240 milliunits, lane 3; 360 milliunits, lane 4; 480 milliunits, lane 5).
identified at 300 nM (Fig. 3B).

Alternatively, when affinity cross-linking, another method for testing binding ability, was performed with 125I-IGF-I or 125I-IGF-II and concentrations of rh-mac25 similar to those used in WLB, an approximately 34-kDa band was detected on the SDS-PAGE gel (Fig. 3, C and D), indicating the 27-kDa rh-mac25 bound to 7-kDa 125I-IGF-I (Fig. 3C, upper panel) or 125I-IGF-II (Fig. 3C, lower panel, and D). Binding of rh-mac25 to IGFs is readily detectable by affinity cross-linking at a mac25 concentration as low as 2 nM. Furthermore, the data indicate that rh-mac25 binding to IGFs is specific, as shown by competition with unlabeled IGF-I and IGF-II, but not with [QAYLL]IGF-II, which has approximately 100-fold less affinity for IGFBPs. We estimated that the affinity of rh-mac25 is at least 5–6-fold lower for IGF-I and 20–25-fold lower for IGF-II than those of rhIGFBP-3E. coli (shown as an approximately 36-kDa band), after determining band densities by densitometer.

Expression of IGFBP-7 mRNA in Normal Human Tissues and Cancer Cells—We next investigated the distribution of IGFBP-7 in normal human tissues and human cancer cells, using Northern blot analysis. As shown in Fig. 4A, the 1.1-kilobase IGFBP-7 mRNA was detected in a broad spectrum of normal tissues. In particular, high expression of IGFBP-7 was observed in small intestine, colon, ovary, prostate, testes, spleen, heart, kidney, and pancreas. Interestingly, expression of IGFBP-7 mRNA was reduced in ER-positive breast cancer and cancer cells from prostate, colon, and lung, although ER-negative breast cancer cells and glioblastoma cells showed relatively high expression (Fig. 4B).

**DISCUSSION**

IGFBPs bind to IGF peptides with high affinity, ranging from 10^{-11} to 10^{-9} M and regulate the biological activities of the IGFs. Amino acid sequence analysis has revealed that relatively high similarity (50–60%) exists among human IGFBPs (1). The most striking feature is a cluster of 18 conserved cysteines found in all IGFBPs except IGFBP-6, which contains 16. Twelve are in the NH2-terminal one-third of the molecule (10 in IGFBP-6) and the remaining six are in the COOH-terminal third of the protein. These terminal regions are highly homologous among the IGFBPs and are speculated to contribute to binding of IGFs. In regard to this sequence homology and striking conservation of cysteines among the IGFBPs, mac25 meets structural criteria as a new member of the IGFBP family by the fact that mac25 contains critical conserved sequences, including the IGFBP motif (GCGCXXC) in the NH2 terminus.
as well as 11 conserved cysteines in the NH\textsubscript{2} terminus and, possibly, 1 at the COOH terminus. Indeed, our affinity cross-linking data showed that mac25 specifically binds IGF-I and IGF-II, indicating that mac25 is a bona fide IGFBP, and can be properly called IGFBP-7. Specificity of the IGFBP-7 binding to IGF was further demonstrated by the relatively low affinity for [QAYLL]IGF-II, an IGF-II analog whose affinity for IGFBPs is 100-fold less than that of native IGF-II.

Compared with IGFBP-3, the affinity of IGFBP-7 for IGFs was assessed by competitive affinity cross-linking to be 5–25-fold lower, which might be attributed to a lack of conserved cysteines, of which only one may be conserved. The COOH terminus of the IGFBPs clearly contributes to the structural configuration required for IGF binding and may even be capable of independent binding of IGF peptides. The failure to demonstrate IGFBP-7 binding of IGF by conventional Western ligand blotting methods may be attributable to the loss of structural integrity under denaturing conditions, since binding was observed when blotting was performed with non-denaturing gels. Alternatively, the affinity of IGFBP-7 for IGF peptides may be underestimated by cross-linking and may, in fact, be 2 or even 3 orders of magnitude lower than that of IGFBP-3. Similar problems have been observed in studies of proteolytic fragments of IGFBP-3, which bind poorly by Western ligand blot, but are readily identified by cross-linking. Nevertheless, even if the affinity of IGFBP-7 proves to be 100-fold lower than that of IGFBP-3, it would still be in a range capable of modulating the interaction of IGFs with their receptors, as is the case with other IGFBPs.

Recent studies from our laboratory have demonstrated that IGFBP-3, the major IGFBP species in the circulation, has a novel growth inhibitory action mediated through its own receptor (IGF-independent action), in addition to regulating IGF access to IGF receptors (IGF-dependent action) in human breast cancer cells (9, 10). Furthermore, transcriptional regulation of IGFBP-3 expression provides a mechanism for both TGF-β2 and all-trans-retinoic acid (all-trans-RA) inhibition of breast cancer cell growth (11, 12). Previous studies have reported that expression of mac25 mRNA was elevated in senescence mammary epithelial cells, while apparently down-regulated in mammary carcinoma cell lines (13, 14). Moreover, mac25 mRNA was up-regulated in normal, growing mammary epithelial cells by all-trans-RA, suggesting that mac25 may be a downstream effector of the RA-induced senescence pathway (14). Taken together with our data showing reduced levels of mac25 (IGFBP-7) mRNA in cancer cells from breast, prostate, colon and lung, it is tempting to speculate that mac25 (IGFBP-7) functions not only as an IGF binding protein, but also as a direct growth-suppressing factor, with an IGF-independent action similar to that of IGFBP-3 (9–12). Additionally, we propose that IGFBPs 1–7 will prove to be members of a superfamily of growth-regulating factors that share structural and sequence homology and which may modulate growth by IGF-dependent and/or IGF-independent mechanisms. Further assessment of IGF binding affinity and the mechanism(s) for the growth-suppressing actions of IGFBP-7 is currently underway.

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