PLIF, a Novel Human Ferritin Subunit from Placenta with Immunosuppressive Activity*

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Ferritin is a ubiquitous iron storage protein existing in multiple isoforms composed of 24 heavy and light chain subunits. We describe here a third ferritin-related subunit cloned from human placenta cDNA library and named PLIF (placental immunomodulatory ferritin). The PLIF coding region is composed of ferritin heavy chain (FTH) sequence lacking the 65 C-terminal amino acids, which are substituted with a novel 48 amino acid domain (C48). In contrast to FTH, PLIF mRNA does not include the iron response element in the 5′-untranslated region, suggesting that PLIF synthesis is not regulated by iron. The linkage between the FTH and C48 domains created a restriction site for EcoRI. PLIF protein was found to localize in syncytiotrophoblasts of placentas (8 weeks of gestation) at the fetal-maternal interface. Increased levels of PLIF transcript and protein were also detected in the breast carcinoma cell lines T47D and MCF-7 but not in the benign corresponding cell line HBL-100. In vitro, PLIF was shown to down-modulate mixed lymphocyte reactions and to inhibit the proliferation of peripheral blood mononuclear cells stimulated with OKT3. The accumulated data indicate that PLIF is an embryonic immune factor involved in down-modulating the maternal immune recognition of the embryo toward anergy. This mechanism may have been adapted by breast cancer cells over expressing PLIF.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY033611.

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The abbreviations used are: H, heavy; L, light; PLIF, placental immunomodulatory ferritin; IRE, iron response element; UTR, untranslated region; mAb, monoclonal antibody; PLIF, placental isoferritin; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactoside; PBMC, peripheral blood mononuclear cell; LC, lymphocyte culture; MLC, mixed lymphocyte culture; PL, proliferation index.

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The RNA Northern blot membrane of CLONTECH (Palo Alto, CA) was hybridized with a 32P-labeled cDNA probe corresponding to the 5′ PstI fragment of human ferritin heavy chain (16). Screening was carried out under low stringency (hybridization at 30% formamide, 6× SSC, and 0.1% SDS, 42 °C). Positive clones were isolated, purified, and analyzed by EcoRI restriction enzyme digest and DNA sequencing. The PLIF clone, which gave a doublet in EcoRI digest analysis, was amplified by PCR using forward and reverse primers flanking the cloning site of the library (F-primer: 5′-GGTGGCGGACTCTGGAGGCCG-3′ and R-primer: 5′-TTGACAACGACCACTGTTGAATG-3′). The PCR product was subcloned into PBuescript cloning vector and analyzed by DNA sequencing.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of PLIF—Human placenta (40 weeks) gt11 cDNA library (CLONTECH) was screened using 32P-labeled cDNA probe corresponding to the 5′ PstI fragment of human ferritin heavy chain (16). Screening was carried out under low stringency hybridization at 30% formamide, 6× SSC, and 0.1% SDS, 42 °C. Washing was carried out under low stringency (hybridization at 30% formamide, 6× SSC, and 0.1% SDS, 42 °C). Positive clones were isolated, purified, and analyzed by EcoRI restriction enzyme digest and DNA sequencing. The PLIF clone, which gave a doublet in EcoRI digest analysis, was amplified by PCR using forward and reverse primers flanking the cloning site of the library (F-primer: 5′-GGTGGCGGACTCTGGAGGCCG-3′ and R-primer: 5′-TTGACAACGACCACTGTTGAATG-3′). The PCR product was subcloned into PBuescript cloning vector and analyzed by DNA sequencing.
induction with IPTG (isopropyl-β-D-thiogalactopyranoside) and lysed in Triton X-100-based lysis buffer. Then, fusion proteins were absorbed from lysates using glutathione-Sepharose 4B beads (Amersham Biosciences) and subsequently eluted with excess of free glutathione. After dialysis, fusion proteins were cleaved by Factor Xa (Amersham Biosciences). Purified PLIF and C48 were obtained by removal of cleaved GST part using glutathione-Sepharose beads.

**Western Blotting**—Aliquots of transformed bacterial cultures were harvested before and after induction with IPTG, lysed with Laemmli sample buffer, and run on 10% SDS-PAGE. Part of the gels were stained with Coomassie Blue. Other gels were blotted onto nitrocellulose membranes and reacted with CM-H-9 mAb (3). Specific bands were detected with horseradish peroxidase anti-mouse IgG (Sigma) and ECL chemilluminescence (Amersham Biosciences).

**Preparation of Rabbit Anti-C48 Sera**—Rabbits were immunized with Pure C48 (50 μg of purified protein per rabbit mixed v/v with complete Freund’s adjuvant). Each rabbit was immunized on days 1, 7, and 21. On day 28, rabbits were bled and immunoglobulins (Ig) were isolated. Immunohistology—PLIF expression was analyzed by immunohistochemistry performed on paraffin sections of human placenta (8 weeks of gestation) and on ethanol-fixed breast cell lines HBL-100, T47D, and MCF-7. Staining was performed using rabbit anti pure C48 Ig (1:150 dilution) followed by a secondary peroxidase-conjugated anti-rabbit IgG reaction and development with stable diaminobenzidine and Gill’s hematoxylin counter stain. As a control, a parallel staining was carried out with anti-C48 Ig preabsorbed with C48-GST. For preabsorbtion experiments, the anti-C48 antibody dilution was preincubated for 1 h with C48-GST bound to glutathion-Sepharose-4B beads (Amersham Biosciences). The beads were removed by microcentrifugation. Placenta villous tissues were collected from healthy pregnant women undergoing elective termination of a normal pregnancy.

**Lymphocyte and Mixed Lymphocyte Cultures**—Human peripheral blood mononuclear cells (PBMCs, responder and stimulator) were obtained from healthy blood bank donors. Responder PBMCs were cultured alone (LC) or mixed with irradiated allogeneic stimulator cells at 1:1 ratio (MLC). For culture treatments, 1 μg/ml purified PLIF or C48 was added to cultures immediately after responder cell plating. These experiments were carried out in 96-well round bottom tissue culture plates in RPMI 1640 culture medium containing 10% human AB serum. At day 4 of the experiment, cells were pulsed with 1 μCi/well of [3H]thymidine and harvested 16 h later. Proliferation index (PI) of lymphocyte cultures was calculated as counts/min (cpm) in stimulated MLC divided to cpm of unstimulated LC for each specific treatment. In anti-CD3 (OKT3) experiments, human mononuclear cells were treated with different concentrations of C48 or not treated and subsequently stimulated with anti-CD3 (100 ng/ml). After 48 h, cultured cells were pulsed with 1 μCi/well of [3H]thymidine and harvested 16 h later.

**RESULTS**

**Cloning of PLIF cDNA**—We screened a human placenta cDNA library using a cDNA fragment corresponding to the 5′ PstI fragment of human ferritin heavy chain (16). Hybridization was carried out under low stringency conditions. More than one-hundred positive clones were picked up. Excision of inserts from positive clones with EcoRI digest revealed single inserts of up to 0.9 kb in most clones except one, which exhibited a doublet of about 0.4 kb. DNA sequencing of all the 0.9-kb single inserts revealed normal H ferritin sequences. Furthermore, we analyzed the single clone that exhibited a doublet band following EcoRI digest, knowing that there is no restriction site for EcoRI within H ferritin sequence. Thus, we obtained the full-length insert of this clone by PCR
amplification using Agt11 forward and reverse primers flanking the cloning site of the library. The PCR product of 0.9 kb was purified, subcloned into a cloning vector, and analyzed by DNA sequencing. The DNA sequence revealed a novel molecule, which was later designated “placental immunomodulatory ferritin” (PLIF).

DNA sequence analysis of the PLIF cDNA clone exhibited an uninterrupted open reading frame encoding a novel protein of 165 amino acids with a predicted molecular mass of 22 kDa (Fig. 1A). The predicted amino acid sequence revealed that 117 amino acids from the N terminus share the highest homology (100%) with human ferritin heavy chain, whereas the C-terminal 48 amino acids (C48) represent a novel sequence with no known matching sequences (Fig. 1, A and B). The predicted polypeptide length of PLIF is close to that of H ferritin (165 and 182 amino acids, respectively) (17). In the nucleotide sequence level, the identity with H ferritin starts 22 bp upstream of the initiating ATG and ends at bp 351 from the open reading frame. It is noteworthy that the EcoRI site exists at the linkage point between the ferritin and C48 domains of PLIF cDNA. On the other hand, the IRE is missing in PLIF mRNA and is substituted by other sequences, suggesting that PLIF synthesis is not regulated by iron as for H ferritin.

**PLIF Transcript in Tissues and Cell Lines**—Northern blot analysis of mRNA from various human tissues using a probe corresponding to the 3′-UTR (PLIF exclusive) showed that the mRNA corresponding to PLIF is of 0.9 kb (Fig. 2A). Variations in the level of PLIF transcripts were observed in different tissues (Fig. 2A).

To test whether PLIF transcripts are up-regulated in malignant breast cells as described for p43 (PLF), Northern blot analysis was performed on total RNA from T47D and MCF-7 breast carcinoma cell lines and HBL-100 immortalized breast epithelial cell line. As shown in Fig. 2B, an increased level of PLIF mRNA was exhibited in T47D and MCF-7 cells compared with very low levels in HBL-100 cells.

**Expressed PLIF Is Immunologically Related to PLF**—We subcloned the full-length coding region of PLIF and its C48 domain into p-GEX prokaryotic expression vector. Both proteins were expressed in *E. coli*, as GST fusion proteins as we show in SDS-PAGE (Fig. 3A). Analysis by SDS-PAGE and

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**Fig. 2.** PLIF mRNA expression in human tissues and cell lines. A, Northern blot analysis of mRNA from various human tissues with 3′-UTR probe from PLIF. B, Northern blot analysis of total RNA from T47D, MCF-7, and HBL-100 cell lines with the same probe. The relative amounts of mRNA in each lane were determined using the β-actin probe.

**Fig. 3.** Expression of recombinant human PLIF and C48. A, SDS-PAGE of GST-C48 and GST-PLIF fusion proteins compared with GST alone, stained with Coomassie Blue. Specific bands were demonstrated only after IPTG induction (+). B, Immunoblot of the above fusion proteins and PLF as a positive control, using anti-PLF CM-H-9 mAb.
protein immunoblotting, using anti-PLF mAb CM-H-9 (9), revealed that both recombinant PLIF and C48, but not GST, reacted with CM-H-9 mAb (Fig. 3B). This result indicated that PLIF is immunologically related to PLF and that the specific immunogenic epitope of PLF is derived from the novel C48 amino acid sequence. Moreover, the ferritin-like domain of PLIF also indicates that this factor is related to PLF.

Localization of PLIF in Human Placenta—To further clarify the possible role and mechanism of action of PLIF, we determined its subcellular localization in sections of human placenta (8 weeks of gestation) by immunostaining with antibodies to pure C48 antibodies. PLIF was found to localize in the syncytiotrophoblasts and Hofbauer cells (embryonic mononuclear cells) within the intermediate villi (Fig. 4A). This immunoreactivity was removed by preabsorption (Fig. 4B). This pattern was similar to that observed with anti-PLF mAb CM-H-9 (11).

Expression of PLIF Protein in Breast Cell Lines—Immunohistochemical staining of breast cell lines with pure anti-C48 Ig revealed cytoplasmic staining in both breast cancer cell lines T47D and MCF-7 (Fig. 5, A1 and B1). Consistent with it being specific antiserum staining was preabsorbed with the immunizing peptide (Fig. 5, A2 and B2). In comparison, only background staining was detected in HBL-100 breast epithelial cells reacted with anti-C48 Ig (Fig. 5C1), which was comparable with that with preabsorbed anti-C48 Ig (Fig. 5C2). These results correlated well with the level of PLIF transcripts in these cells.

PLIF Is Immunosuppressive in Vitro—PLIF and C48, cleaved and purified from their GST fusion proteins, were tested to determine whether they exhibit immunomodulatory activity similar to PLF. Both significantly reduced the lymphocyte proliferation in allogeneic MLC, as indicated by decreased proliferation index (PI = 4) of the treated cultures compared with that of nontreated controls (PI = 16) (Fig. 6A). These results indicate that PLIF, like PLF, is immunomodulatory, and C48 represents its bioactive domain. Moreover, we demonstrated marked immunosuppressive activity of C48 on anti-CD3 (OKT3)-activated human PBMCs (Fig. 6B).

**Fig. 4.** Localization of PLIF in intermediate villous of human placenta (8 weeks of gestation). A, immunohistology using rabbit anti-C48 Ig depicting expression of PLIF in syncytiotrophoblast cells (arrow) and in Hofbauer cells (arrowhead) (>400). B, the immunoreactivity was removed by preabsorption with C48-GST (>400).

**Fig. 5.** Expression of PLIF protein in breast cell lines detected by anti-C48 Ig. Cytoplasmic immunostaining is depicted in cancer cell lines T47D (A1), and MCF-7 (B1), and this immunoreactivity was removed by preabsorption (A2 and B2, respectively). Background staining was observed in HBL-100 epithelial cells (C1) similar to that observed with preabsorbed serum (C2) (>400).

**Fig. 6.** Effect of PLIF and C48 on human lymphocyte cultures. A, PI for allogeneic stimulation (MLC) of human mononuclear cells treated with PLIF, C48, or untreated. B, thymidine incorporation into anti-CD3 (OKT3)-stimulated human mononuclear cell cultures pretreated with C48 or cell cultures that were not treated.
PLIF, a Novel Immunosuppressive Placental Ferritin Subunit

DISCUSSION

In this report, we introduce a new human ferritin subunit cloned from placenta and designated PLIF. One of the fascinating features of PLIF is its unique molecular structure: a fusion of apparently two unrelated components to compose a bioactive molecule. This could be a result of an early recombination event between one of the several ferritin heavy chain genes (17) with another, yet unknown, gene(s).

The difficulty in isolating PLIF cDNA may refer to several reasons. First, the fusion of the ferritin-like domain and the C48 domain created a restriction site for EcoRI, a site that does not exist in the DNA sequence of ferritin heavy chain. The EcoRI site is commonly used as a linker in constructing cDNA libraries. Thus, such a site will become deceptive when unexpectedly detected in the middle of known genes such as in ferritin heavy chain. Second, PLIF transcripts exist at very low copy number, compared with ferritin heavy chain, in placental tissues at term delivery (40 weeks) as we observed in the placenta cDNA library screen and the Northern blot analysis. It is noteworthy that in the current study PLIF expression was exhibited by immunohistochemical staining in syncytiotrophoblast cells of human placenta (8 weeks of gestation). This is compatible with the previous report on the expression of PLF (11).

However, it was also shown that PLF is down-regulated in those cells at 17 weeks of gestation up to term (11). This may explain the current observation of the proportionally low PLIF mRNA transcripts exhibited in the tissue mRNA blot, as it was obtained from term placenta. Third, the size of PLIF mRNA and its translated peptide are overlapping with H ferritin and therefore cannot be differentiated by electrophoresis (16, 18).

The data presented in this work shed light on the structural and functional properties of this novel ferritin subunit. Structurally, PLIF lacks the IRE, suggesting that its synthesis, in contrast to H and L ferritin subunits, is not regulated by iron. For the H, but not L, subunit, it was reported that cell differentiation and other factors might regulate its transcription (19). Likewise, PLIF acting as an immunomodulatory factor may be regulated by differentiation processes in the appropriate cells. There is a need to carefully analyze the 5'-UTR of PLIF and identify possible regulatory elements other than IRE.

The immunosuppressive characteristic of PLIF is demonstrated in two immune activation models in vitro, MLC and anti-CD3 stimulation. Furthermore, PLIF was localized in the placenta at fetal-maternal interface and in the immune Hofbauer cells.

Taken together, the localization and activity of PLIF suggest its possible involvement in the modulation of the maternal immune recognition of embryo toward anergy.

The increased level of PLIF transcripts and its protein expression observed in T47D and MCF-7 breast carcinoma cell lines, but not in the breast epithelial cell line HBL-100, suggest that PLIF may function as an immunosuppressive protein enabling immune escape of the developing breast cancer cells, as has been suggested previously for PLF (8).

It was shown that the immunomodulatory activity of PLIF is related to the C48 domain irrespective to ferritin domain. C48 is proline-rich, which may indicate that it functions through binding to Src homology 3 domains of signaling molecules in target cells (20). It even contains the consensuse Src homology 3 binding motif, PXKP (21) (P, proline and X, any amino acid) at position 121–124 of the PLIF polypeptide (Fig. 1). C48 contains also an Arg-Arg (RR) cellular kinase-binding motif that is important for binding to serine/threonine kinases (22).

There is a similarity between PLF and PLIF in their immunological cross-reactivity with CM-H9 mAb, placenta expression, and immunosuppressive function. Moreover, the p43 subunit of PLF may represent a dimer of PLIF (22 kDa). With such characteristics, PLIF could become a therapeutic modality in high risk pregnancies associated with low levels or deficiency of PLF (10–12). Furthermore, PLIF and its active domain C48 may be developed into an immunosuppressive therapeutic factor for treatment of various immune-related disorders.

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