Molecular Characterization and in Vitro Biological Activity of Placentin, a New Member of the Insulin Gene Family*  

(Received for publication, May 30, 1996) 

Ahmet Koman§, Sylvie Cazaubon¶, Pierre-Olivier Couraud¶, Axel Ullrich, and A. Donny Strosberg†  

From the *Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 82512 Martinsried, Federal Republic of Germany and the ¶Laboratoire d’Immuno-Pharmacologie Moléculaire, Institut Cochin de Genétique Moléculaire, CNRS UPR 0415, Université Paris VII, 22 rue Méchain, 75014 Paris, France  

Insulin and insulin-like growth factors belong to a family of polypeptides involved in essential physiological processes. Placentin, a new member of the insulin family, was recently identified as a 139-amino acid open reading frame from a cDNA clone isolated from a subtracted library of first trimester human placenta. Tris/Tricine/SDS-polyacrylamide gel electrophoresis and immunoblot analyses of histidine-tagged recombinant placentin indicate that it is composed of two peptide chains of apparent molecular masses of 4 and 13 kDa. Conditioned media produced by recombinant expression of placentin cDNA in the placental 3AsubE cell line were assayed for biological activity and found to stimulate tyrosine phosphorylation and DNA synthesis. While these effects closely mimicked those of insulin, they were not mediated by the insulin receptor as shown by the lack of tyrosine phosphorylation of this receptor upon placentin treatment. Moreover, in cytotrophoblast primary culture, production of chorionic gonadotropin, a marker of trophoblast differentiation, was increased upon treatment with placentin-conditioned media, while unaffected by insulin. These results suggest that placentin might participate in the cellular proliferation and/or differentiation processes during placental development.

Insulin and insulin-like growth factors belong to a family of polypeptides essential for proper regulation of physiological processes such as energy metabolism, cell proliferation, development, and differentiation (1, 2). These polypeptides exert their effects through membrane receptors belonging to the superfamily of polypeptide growth factor receptor tyrosine kinases and their oncogenic analogs (3–5). The intracellular signal transduction pathway for these receptors is characterized by a tyrosine kinase activity that initiates a chain of phospho-  

© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.

THE JOURNAL OF BIOLOGICAL CHEMISTRY  
Vol. 271, No. 34, Issue of August 23, pp. 20238–20241, 1996  
© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.  
Printed in U.S.A.
Expression and Molecular Characterization of Recombinant Placentin—Placentin displays a number of structural features resembling those of insulin (Fig. 1A), including the characteristic six half-cystine residues of the insulin family and 25% amino acid sequence identity to insulin A- and B-chains in corresponding domains. Dibasic recognition sites for putative enzymatic cleavage between C and A domains are also conserved, whereas no homology is found in respective cleavage sites between B and C domains. The consensus sequence Arg-X-Lys-Arg between the C and A domains is a furin recognition site that would allow processing to occur in most cells (20) (see Fig. 1A).

The molecular characterization of placentin was undertaken using recombinant placentin. Transfection experiments indicated that human kidney 293, monkey kidney COS-7, and human placental 3AsubE cells were able to produce bioactive placentin as assayed by induction of tyrosine phosphorylation of cellular proteins described below. In order to produce and purify significant amounts of placentin, the cDNA sequence was modified, adding a carboxyl-terminal six-residue histidine tag to the recombinant peptide, and was expressed in human kidney 293 cells known to show high transfection and production efficiency. The modified peptide was found to be secreted and was purified from conditioned media by nickel-agarose chromatography. Analysis of the recombinant product by Tris/TricineSDS-PAGE (Fig. 1B) indicated the presence of two major subunits of 4 and 13 kDa that could correspond to A- and B-chain subunits, respectively. Western blot analysis using antibodies generated by immunizing rabbits with recombinant glutathione S-transferase-placentin fusion protein revealed immunoreactive peptides of similar size (Fig. 1C).

Stimulation of Tyrosine Phosphorylation—The biological activity of recombinant placentin was investigated using 3AsubE cells derived from trophoblasts of terminal placenta by temperature-sensitive SV40 immortalization. They have been described as maintaining the trophoblast phenotype at nonpermissive temperature (37–40°C) (15). Treatment of 3AsubE cells with conditioned media from stable placentin sense cDNA-transfected 3AsubE cells or with insulin-induced rapid (10 min) tyrosine phosphorylation of several proteins of apparent molecular masses of about 60, 85, 95, 120–140, and 170–190 kDa (Fig. 2A). Densitometric scan analysis revealed that...
placentin- and insulin-induced similar increases in tyrosine phosphorylation of the 85-kDa phosphoprotein (2.4- and 2.9-fold, respectively) (Fig. 2C) while some proteins phosphorylated after insulin treatment were not significantly affected by placentin (e.g. around 150 kDa). Comparable results were obtained with conditioned media from cells transiently expressing placentin; treatment with conditioned media from nontransfected or vector-transfected cells gave basal phosphorylation levels equivalent to that shown for media from antisense-transfected cells (Fig. 2A). In addition, conditioned media from His-tagged placentin expressing 293 cells was found to have similar activity in stimulation of tyrosine phosphorylation as that shown for 3AsubE-produced placentin-conditioned media in Fig. 2A. In preliminary experiments, stimulation of tyrosine phosphorylation with purified His-tagged placentin was observed as well, indicating that the observed activity is a direct effect of placentin.

Lack of Insulin Receptor Activation—Since cross-reactivity on insulin receptors has been described for other members of the family (21), immunoprecipitation with anti-IR antibodies was performed in order to investigate the possibility that placentin effects were mediated by insulin receptors (Fig. 2D). While the IR β-subunit was found to be tyrosine-phosphorylated in response to insulin as expected, no detectable phosphorylation was observed upon placentin treatment, suggesting lack of IR activation in the response to placentin.

Increase of \[^{3}H\]Thymidine Incorporation in Placentin-treated 3AsubE Cells—Increase of tyrosine phosphorylation is often involved in the stimulation of DNA synthesis in many different cell types. The biological effects of placentin were also investigated by measuring stimulation of \[^{3}H\]thymidine incorporation in quiescent 3AsubE cells after treatment with conditioned media from placentin cDNA-transfected cells (Fig. 3). Stimulation of similar magnitude to that induced by insulin was observed in response to placentin (increased incorporation by 1.8-fold for placentin and 1.7-fold for insulin).

Stimulation of hCG Production in Primary Cultures of Trophoblasts by Placentin—Further characterization of placentin effects was performed on trophoblasts in primary culture. Trophoblasts differentiate to form syncytiotrophoblasts throughout placental development until parturition and the production of hCG is considered as a marker of this differentiation, also in vitro (22). Treatment with conditioned media from sense cDNA-transfected 3AsubE cells increased hCG production 2.6-fold, suggesting a stimulated differentiation process, while conditioned media from antisense transfected cells had no effect (Fig. 4). In agreement with previous investigations (23), insulin did not affect hCG production by trophoblasts in primary culture.

DISCUSSION

The present study describes the molecular characterization and the in vitro biological activity of placentin, a new member of the insulin gene family. Other members of the insulin family require various posttranslational modifications for bioactivity. For insulin and relaxin (6) the leading signal peptide as well as the connecting C peptide are excised by tissue-specific posttranslational processing from prepro-sequences, leaving two peptide chains A and B linked by disulfide bridges, whereas the insulin-like growth factors are processed without removal of the C peptide (21). For recombinant placentin expression, 293, COS-7, and 3AsubE cells were compared and found to secrete bioactive product after transfection. When placentin was expressed in 293 cells, the two immunoreactive peptide chains of 4 and 13 kDa observed may derive from cleavage between the C and A domains, as expected from the sequence data upon furin cleavage. 3AsubE cells derived from trophoblasts of terminal placenta were selected as host cells for transfection with unmodified cDNA when producing placentin-conditioned media for characterization of biological activity. These conditions presumably meet those for the native peptide most closely.

Purification of bioactive His-tagged placentin was found to be difficult. Thus, purification on a larger scale of the peptide (whether recombinant or native) requires further optimization in terms of solubility and bioactivity.

Tyrosine phosphorylation of cellular proteins is recognized as a key factor in the processes leading to cell proliferation and/or differentiation. A large number of membrane receptors as well as cytosolic protooncogene products have been identified as tyrosine kinases mediating the biological activity of polypeptide growth factors, such as insulin. The tyrosine phosphorylation and \[^{3}H\]thymidine incorporation stimulating activity of recombinant placentin was investigated on 3AsubE
cells, assuming that they might express receptors binding placentin. Autocrine regulation is one of the characteristic features of trophoblasts and placenta has been described to express several types of insulin-related receptors (24–26). The effects of placentin-conditioned media closely mimicked those of insulin both qualitatively and in magnitude, strongly suggesting that placenta could activate signal transduction pathways generally associated to polypeptide growth factor receptors. However, in contrast to insulin, placentin did not induce the autophosphorylation of the insulin receptor β-subunit. Consistently, insulin did not affect hCG production under the experimental conditions used, nor was this described for any other member of the insulin family. These observations suggest that placenta might be acting through a receptor positively coupled to cellular tyrosine phosphorylation, distinct from the insulin receptor. The possibility exists, however, that some of the observed effects with conditioned media might be indirect effects of placentin, through specific induction of other compounds.

In summary, the biological activity on placental cells of a new member of the insulin family was demonstrated in vitro. Present results suggest that placentin is expressed by, and affects, trophoblasts and thus might be involved in an autocrine loop, joining IGF-II, EGF, and PDGF in concerted regulation of placental development and growth. The placenta is well defined as an endocrine organ producing several polypeptides with maternal and fetal targets. While the activity defined in this study was restricted to placental cells, preliminary data suggest possible effects on cells of other tissue origin. Placentin might thus have endocrine/paracrine effects in maternal or fetal compartments depending on its bioavailability, receptor distribution and selectivity.

Acknowledgments—We thank Eliane Alsat, Cordula Ebner von Eschenbach, Robert J., Gillies, Jean-François Giot, Evgeny N. Imyanitov, and Daniele Saltarelli for their valuable contributions.

REFERENCES
1. Baker, J., Liu, J.-P., Robertson, E. J., Efstratiadis, A. (1993) Cell 75, 73–82
2. White, M. F. & Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
3. Ullrich, A. & Schlessinger, J. (1990) Cell 61, 203–213
4. Fantl, W. J., Johnson, D. E. & Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–481
5. Cadena, D. L. & Gill, G. N. (1992) FASEB J. 6, 2332–2337
6. Bryant-Greenwood, G. D. & Schwabe, C. (1994) Endocr. Rev. 15, 5–26
7. Burkhardt, E., Adham, I. M., Brosig, B., Eidner, T., Mattel, M-G. & Engel, W. (1994) Genomics 20, 13–19
8. Blundell, T. L., Bedarida, S., Rinderknecht, E. & Humbel, R. E. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 180–184
9. Holmgren, L., Claesson-Welsh, L., Heldin, C.-H. & Ohlsson, R. (1992) Growth Factors 6, 219–231
10. Ohlsson, R., Glaser, A., Holmgren, L., & Franklin, G. (1993) In The Human Placenta (Redman, C. W. G., Sargent, I. L., and Starkey, P. M. eds) Vol. 1, pp. 33–81, Blackwell Scientific Publications, Oxford
11. Hofmann, G. E., Drews, M. R., Scott, R. T., Jr., Navet, D., Hefler, D. & Deligdisch, L. (1992) J. Clin. Endocrinol. Metab. 74, 983–988
12. Chassin, D., Beniﬁla, J.-L., Delattre, C., Fernandez, H., Ginisty, D., Janneau, J.-L., Prade, M., Contesso, G., Caillou, B., Tournaire, M., Frydman, R., Elias, D., Bedossa, P., Bidart, J.-M., Bellet, D. & Koman, A. (1994) Cancer Res. 54, 5217–5223
13. Chassin, D., Laurent, A., Bellet, D. & Koman, A. (1995) Genomics 24, 465–470
14. Schagger, H., & von Jagow, G. (1987) Anal. Biochem. 166, 368–379
15. Chou, J. Y. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 1409–1413
16. Cazaubon, S., Parker, P. J., Strosberg, A. D. & Coureaud, P. O. (1993) Biochem. J. 293, 381–386
17. Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Tartare, S. & Van Obberghen, E. (1993) J. Biol. Chem. 268, 7358–7364
18. Bayne, M. L., Cacchione, R., Kelder, B., Applebaum, J., Cicchi, G., Shiaprio, J.-A., Paskalev, F. & Kopchick, J. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2633–2642
19. Alsat, E., Haziza, J. & Evain-Brion, D. (1993) J. Cell. Physiol. 154, 122–128
20. Groskreutz, D. J., Slivkowsky, M. X. & Gorman, C. M. (1994) J. Biol. Chem. 269, 6241–6246
21. Lebovitz, D., Basser, J. C., Tuchman, B. & Grossman, A. I. (1993) J. Clin. Endocrinol. Metab. 72, 1383–1390
22. Kliman, H. J., Nestler, J. E., Sermad, E., Sanger, J. M. & Strauss, J. F., III (1988) Endocrinology 118, 1567–1582
23. Ren, S.-G. & Braunstein, G. D. (1991) Endocrinology 128, 1623–1629
24. Ritvos, O., Rutanen, E.-M., Pekonen, E., Jalkanen, J. & Suikkari, A.-M. & Ranta, T. (1989) Endocrinology 122, 395–401
25. Zhang, B. & Roth, R. A. (1992) J. Biol. Chem. 267, 18320–18328
26. Jonas, H. A., Newm, J. D. & Harrison, L. C. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4124–4128

Fig. 4. Effects of placentin treatment on the production of hCG by trophoblasts during their third day in primary culture. Relative ratios to control values are presented as histograms with standard error bars (mean values of four experiments (two for insulin) each in triplicate. Lanes correspond to the following treatments: 1 = control (medium only); 2 = conditioned media from antisense placentin cDNA-transfected 3AsubE cells; 3 = conditioned media from sense placentin cDNA-transfected 3AsubE cells; 4 = 170 nM insulin.