Secretion of Proliferin*

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ABSTRACT. Proliferin (PLF) is a member of the PRL-GH family whose mRNA has been detected in proliferating mouse cells in culture and in mouse placenta. Suspensions of murine placental tissue have been shown to produce PLF detectable by immunoprecipitation. Here we report that PLF is secreted as a heterogeneous glycoprotein by growing mouse cell lines and placental tissue and that the first 29 amino acids of the preprotein serve as a signal peptide. Placental PLF is immunologically distinguishable from the 2 previously described mouse placental lactogens. (*Endocrinology* **120**: 208–213, 1987)

PROLIFERIN (PLF) is a PRL-related protein identified by Linzer and Nathans (1, 2) from the nucleotide sequence of a cDNA clone derived from growing murine BALB/c 3T3 cells. PLF mRNA is undetectable in serum-starved cells and appears after stimulation with either fetal calf serum or purified platelet-derived growth factor, reaching a peak level at the start of DNA synthesis (1). This mRNA is also expressed at high levels in other proliferating murine cell lines (3) and in mouse placental tissue, predominating in the fetal component of the placenta (4). The nucleotide sequence of PLF cDNA revealed a single open reading frame encoding a protein of about 25K with 31% sequence identity to mouse PRL (2, 5). In addition, the PLF sequence contained a hydrophobic leader sequence resembling a signal peptide as well as four Asn-X-Ser sequences, the consensus signal for N-linked glycosylation (6). As an initial characterization of the PLF protein, we describe the preparation of antiserum directed against recombinant PLF and present evidence that PLF is secreted as a glycoprotein lacking the hydrophobic signal peptide.

Materials and Methods

Resolvase-PLF fusion protein and preparation of anti-PLF serum

The 5′-end of PLF-1 cDNA (2) was modified using oligonucleotides to create a Hind III site preceding the start codon, and the Hinc II site 750 nucleotides downstream from the start

codon was converted to an Eco RI site by ligation with phosphorylated Eco RI linkers (New England Biolabs, Beverly, MA). This 750-nucleotide cDNA fragment was then cloned between a Bst NI site (modified to a Hind III site) 350 nucleotides downstream from the start codon for *E. coli* γ,δ-transposon resolvase and the Eco RI site of pBR322 in plasmid pRR71 (7). The resulting plasmid, pλRRPLF, was used to transform strain N4830 (7) using CaCl₂-mediated DNA uptake (8). Bacterial proteins were labeled with [³⁵S]methionine (New England Nuclear, Boston, MA), at 100 μCi/ml in L-broth at 30 or 43 C for 45 min. Cells were lysed in sodium dodecyl sulfate (SDS) buffer, and labeled proteins were electrophoresed in 12% SDS-polyacrylamide slab gels (9). Unlabeled extracts were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose, and bound to antiserum (1:1000 dilution) and [¹²⁵I] iodoprotein A (New England Nuclear), as described previously (10). For immunization, bands were excised from Coomassie blue-stained polyacrylamide gels, lyophilized, crushed into a powder, rehydrated in 3–5 ml PBS and 1 ml Freund's complete adjuvant (Gibco, Grand Island, NY), and periodically injected sc into female New Zealand White rabbits (Bunnyville, Littletown, PA). Of the 8 rabbits immunized in this way, 2 (supplied by D. Linzer) had previously received injections of a synthetic peptide corresponding to PLF amino acids 199–208, coupled to the carrier keyhole limpet hemocyanin. Although immunization with the peptide resulted in antipeptide antibody, no anti-PLF antibody was detectable in the serum from either animal before injection of the resolvase-PLF fusion protein. These 2 animals were among the 3 responders to the resolvase-PLF fusion protein.

Cell culture

CV-1 cells were grown in Minimum Essential Medium (MEM) (Gibco) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and infected with SV40 virus, as described previously (11). BALB/c 3T3 cells were grown and stimulated as described previously (1). Krebs ascites tumor cells were grown in the peritoneal cavity of BALB/c mice (Charles River,

Wilmington, MA) and harvested 2 weeks after inoculation. A PLF-producing CHO line was grown in MEM supplemented with 10% dialyzed fetal calf serum and 11.5 μg/ml proline; for collection of conditioned medium, the medium was changed to MCDB 302 (12).

Construction of the SV40/PLF recombinant virus

The 5' 750 nucleotides of PLF-1, modified as described above, were cloned between the Hind III site at nucleotide 1493 and the Bam HI site at nucleotide 2533 of SV40 DNA (13), and the resulting recombinant was cloned into the Bam HI site of a pBR322 derivative, pKP45 (generously provided by K. Peden). The recombinant plasmid and pd11136 helper viral DNA (14) were digested with Bam HI, diluted to 2 μg/ml, and religated into circles. CV-1 cells were transfected with an equal mixture of the 2 ligation products using the DEAE-dextran method (15). Virus stocks were harvested at 12 days by freeze-thawing 3 times, followed by extraction with 1 ml chloroform/100 ml lysate.

Labeling of cells and analysis of labeled proteins

CV-1 cells (36–48 h postinfection), BALB/c 3T3 cells, Krebs ascites cells (isolated from the peritoneal cavity of mice), or minced placental tissue (10 or 16 days after the appearance of the vaginal plug) were washed twice with methionine-free Dulbecco's MEM (Gibco) and labeled in 0.5 ml of the same medium containing 100–500 μCi/ml [^{35}S]methionine for 2 h. Tunicamycin was added at 2–5 μg/ml 1 h before labeling. Secreted proteins (supernatant) or cell extracts were immunoprecipitated, as described previously (16), and analyzed on SDS-polyacrylamide gels.

RNA purification and filter hybridization

RNA, prepared from guanidinium thiocyanate lysates of whole cells, was electrophoresed on denaturing formaldehyde agarose gels, transferred to nitrocellulose, and hybridized, as described previously (1).

Amino acid sequence analysis

Purified PLF was reduced and subjected to NH$_2$-terminal sequencing by sequential Edman degradation in the Applied Biosystems (Foster City, CA) protein microsequenator (17).

Results

Preparation of anti-PLF serum

To prepare antigen for the purpose of raising antiserum against PLF, PLF cDNA was joined in frame, 350 nucleotides downstream from the translation initiation site of the γ,δ-transposon-encoded resolvase gene in E. coli plasmid pRR71, which is known to overproduce resolvase protein (7). The resulting plasmid, pλRRPLF (Fig. 1A), was introduced into E. coli strain N4830, which contains the thermolabile cI857 λ-repressor, and synthe- of the resolvase-PLF fusion protein from λP$_L$ was ced by shifting the culture from 30 to 43 C. A protein of the predicted size for the fusion protein was synthesized upon temperature shift in cells harboring the plasmid (Fig. 1B, lane 1), but not in cells bearing a plasmid in which the resolvase coding sequence had been deleted (Fig. 1B, lane 3). The identity of the fusion protein was confirmed by its ability to react with antiresolvase serum.

The fusion protein was excised from preparative SDS-polyacrylamide gels and used to immunize rabbits. A Western blot using serum from one of these rabbits showed that this serum reacted with an antigen corresponding in mobility to the fusion protein (Fig. 1C). The highest titer antiserum was obtained from a rabbit that had previously been immunized with a PLF peptide, as described in Materials and Methods. All of the immunoprecipitations shown below were carried out using this high titer antiserum, which will be referred to as anti-PLF serum.

Test of anti-PLF serum with an SV40/PLF recombinant

To determine whether the putative anti-PLF serum reacted with the PLF gene product, PLF cDNA was cloned into the late region of SV40 in place of the major late protein VP1 to express PLF in cultured monkey cells. The structure of the SV40 recombinant is shown in Fig. 2A. Because this recombinant DNA was only 5.5% smaller than wild-type SV40 DNA, we were able to propagate the recombinant in CV-1 cells as virus particles by using an early region deletion mutant (d11136) (14) as a helper virus. The mixed stock of helper virus and PLF recombinant was then used to infect CV-1 cells at high multiplicity, and newly synthesized proteins were labeled with [^{35}S]methionine.

Figure 2B shows that the anti-PLF serum precipitated a heterogeneous set of labeled proteins ranging from 25–40K from an extract or from the medium of cells infected with the recombinant virus stock (lanes 2 and 6), but not from the extract or medium of cells infected with wild-type SV40 (lanes 1 and 5). In cells pretreated with tunicamycin, an inhibitor of N-linked glycosylation (18), a single major protein of about 22K was immunoprecipitated (lane 8); this is consistent with the size predicted for a fully nonglycosylated proliferin whose signal sequence has been cleaved (see below). Hence, we conclude that the anti-PLF serum reacts with the product of the PLF gene and that PLF is both secreted and N-glycosylated by CV-1 cells infected with the recombinant virus, as predicted from the inferred amino acid sequence of PLF (2).

Secretion of glycosylated PLF by mouse cells in culture

To determine whether PLF is secreted by growing murine cells shown to contain PLF mRNA (1, 3), we immunoprecipitated ^{35}S-labeled secreted proteins from the medium of BALB/c 3T3 cells and Krebs ascites
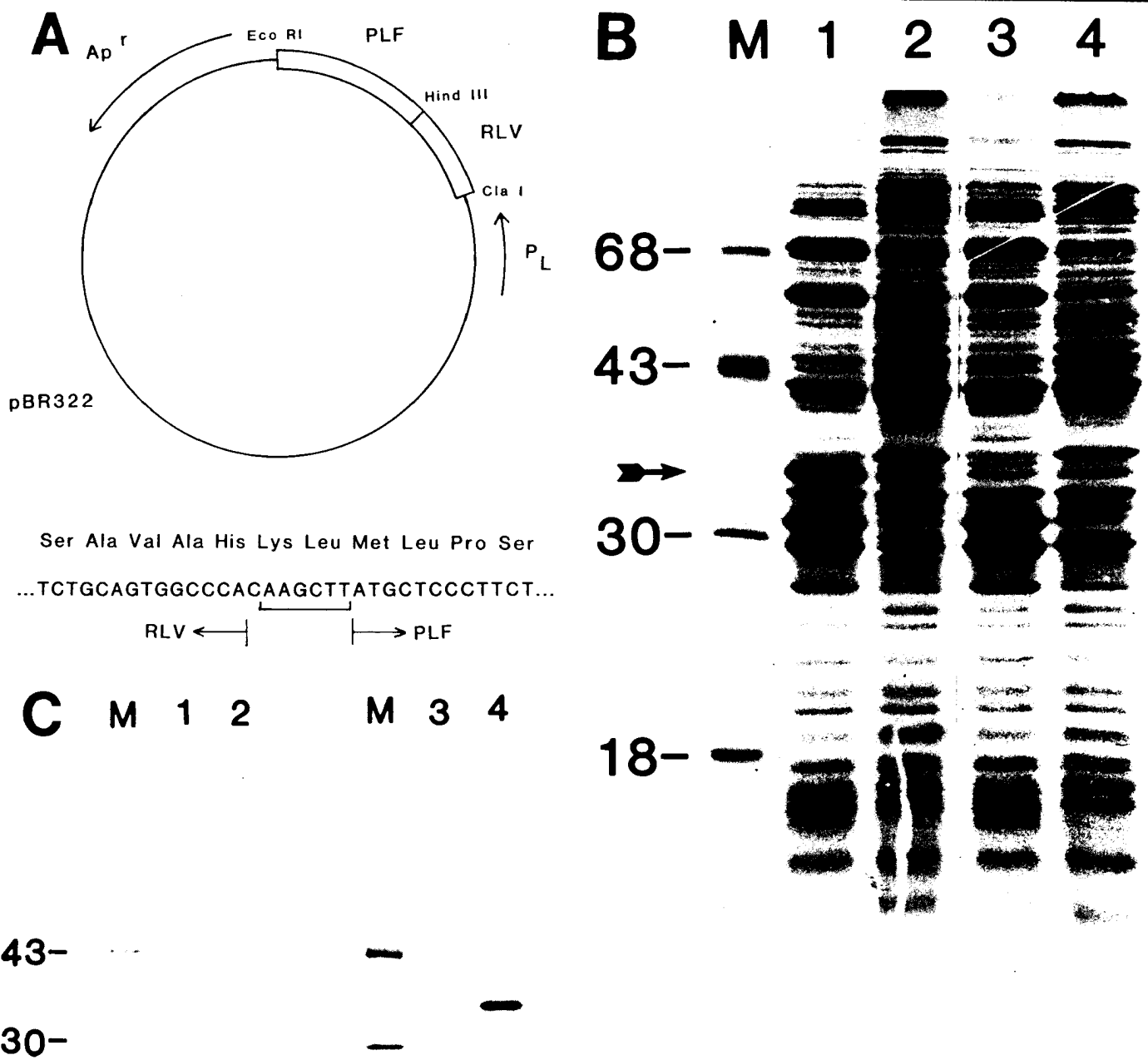
A

Ap r

Eco RI   PLF

Hind III

RLV

Cla I

P_L

pBR322

Ser Ala Val Ala His Lys Leu Met Leu Pro Ser

...TCTGCAGTGGCCCACAAGCTTATGCTCCCTTCT...

RLV ⟵──┤   ├──⟶ PLF

B   M 1 2 3 4

68-

43-

➡

30-

18-

C   M 1 2   M 3 4

43-

30-

FIG. 1. Expression of resolvase-PLF fusion protein. A, Diagram of plasmid pλRRPLF with the sequence of the junction between resolvase (RLV) and PLF coding sequences shown below. The HindIII site is *bracketed*. B, N4830 cells bearing plasmid pλRRPLF (lanes 1 and 2) or pλcII (lanes 3 and 4; derived from pRR71 by removal of the Cla I/Eco RI fragment containing the coding sequence for resolvase) were grown to midlog phase at 30 C and labeled for 45 min with [³⁵S]methionine at 30 C (lanes 2 and 4) or 43 C (lanes 1 and 3). Total cellular extracts were analyzed by SDS-polyacrylamide gel electrophoresis. M, Marker proteins of 68K (BSA), 43K (ovalbumin), 30K (carbonic anhydrase), and 18K (lactoglobulin). The *arrow* indicates the expected position of the resolvase-PLF fusion pr ,tein. C, N4830 cells bearing plasmid pRR71 (lanes 1 and 3) or p RRPLF (lanes 2 and 4) were grown to midlog phase at 30 C and snifted to 43 C for 45 min. Total cellular extracts were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, bound to either preimmune (lanes 1 and 2) or immune (lanes 3 and 4) serum and [¹²⁵I]iodoprotein A, and analyzed by autoradiography. M, Marker proteins.

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A

ori

early late

Hind III

Bam HI PLF

Met Ala Pro

SV40 wt ...AAAAGCTTATGAAGATGGCCCCA...

Met Leu Pro Ser Leu

SV40/PLF ...AAAAGCTTATGCTCCCTTCTTTG...

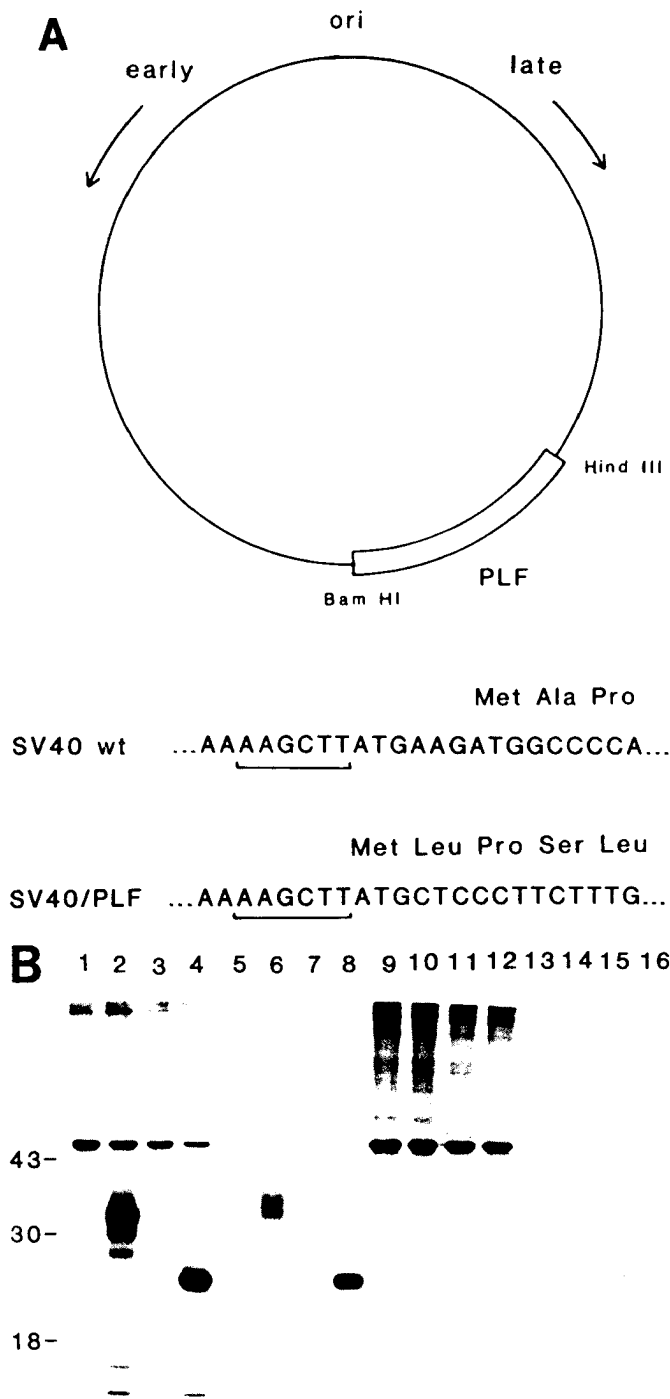**B** 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

43-

30-

18-

FIG. 2. Expression of PLF by an SV40/PLF recombinant virus. A, Diagram of the SV40/PLF recombinant virus with the sequences of wild-type SV40 and the recombinant in the region of the start codons for VP1 and PLF, respectively, shown below. The HindIII site is *bracketed.* B, CV-1 cells infected with either wild-type SV40 (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or a mixed stock of d11136 and the SV40/PLF recombinant (lanes 2, 4, 6, 8, 10, 12, 14, and 16) were pulse labeled for 2 h with [^{35}S]methionine in the absence (lanes 1, 2, 5, 6, 9,10, 13, and 14) or presence (lanes 3, 4, 7, 8, 11, 12, 15, and 16) of 2 μg/ml

tunicamycin. Supernatants (lanes 5–8 and 13–16) or total cell extracts (lanes 1–4 and 9–12) were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with immune serum (lanes 1–8) or preimmune serum (lanes 9–16). *Numbers at left* indicate the positions of marker proteins.

tumor cells using the antiserum described above. As with the CV-1 cells, the anti-PLF serum immunoprecipitated a heterogeneous set of proteins from these cells in the absence of tunicamycin and a discrete protein of 22K from cells exposed to tunicamycin (Fig. 3A). As seen in Fig. 3A, the amount of labeled immunoprecipitable protein was greater when cells were treated with tunicamycin. We interpret this to mean that some of the glycosylated forms of PLF, particularly those secreted by BALB/c 3T3 cells, are poorly recognized by the anti-PLF serum raised against the nonglycosylated fusion protein or that tunicamycin enhances the rate of secretion of PLF. We next examined the time course of PLF synthesis and secretion after stimulation of quiescent 3T3 cells with medium containing 20% fetal calf serum. At various times after serum stimulation, cells were treated with tunicamycin for 1 h and pulse labeled with [^{35}S]methionine for 2 h; the secreted proteins were then immunoprecipitated with anti-PLF serum (Fig. 3B). The peak of proliferin synthesis and secretion was reached approximately 12–18 h after stimulation, which correlated closely with the appearance of the PLF mRNA isolated from cells grown in a parallel set of dishes.

Secretion of PLF by mouse placenta

Linzer *et al.* (4) detected PLF mRNA in mouse placental tissue and showed that minced placentas placed in culture secreted PLF into the medium (they also demonstrated that PLF was immunologically distinct from mouse placental lactogen II). We analyzed the placental secretion of PLF with more potent anti-PLF serum and compared the reacting proteins with those reacting with antisera against mouse placental lactogen I (19) and placental lactogen II (20). Figure 4 shows that the anti-PLF serum detected a variety of forms of glycosylated PLF (lanes 5 and 7) synthesized by placentas from the 10th or 16th day of pregnancy and that PLF is clearly distinct antigenically from both mouse placental lactogens. It is also evident that the times of maximal secretion of PLF and placental lactogen I are different.

Site of cleavage of PLF

To determine the site of cleavage of pre-PLF during the secretory process, we determined the N-terminal amino acid sequence of secreted PLF and compared it to the sequence inferred from the nucleotide sequence of PLF cDNA (2). For this purpose, PLF was purified from the medium of a Chinese hamster ovary cell line that

FIG. 3. Secretion of PLF by cultured mouse cells. A, BALB/c 3T3 cells (lanes 1–12) were labeled for 2 h with [^{35}S]methionine after serum starvation (lanes 1, 2, 7, and 8), after 12 h of serum stimulation (lanes 3, 4, 9, and 10), or during logarithmic growth (lanes 5, 6, 11, and 12) in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 5 µg/ml tunicamycin. Supernatants were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with immune (lanes 1–6) or preimmune (lanes 7–12) serum. Krebs ascites cells (lanes 13–20) were labeled for 2 h with [^{35}S]methionine in the absence (lanes 13, 15, 17, and 19) or presence (lanes 14, 16, 18, and 20) of 2 µg/ml tunicamycin, and supernatants (lanes 13, 14, 17, and 18) or total cell extracts (lanes 15, 16, 19, and 20) were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with preimmune (lanes 13–16) or immune (lanes 17–20) serum. M, Marker proteins. B, BALB/c 3T3 cells were either pulse labeled for 2 h with [^{35}S]methionine in the presence of 5 µg/ml tunicamycin or harvested for RNA preparation at the indicated hours after serum stimulation. Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with anti-PLF serum. Total cellular RNA was electrophoresed, blotted, probed with nick-translated PLF-1, and autoradiographed.

carries amplified PLF cDNA and overproduces the protein (the construction of this line and the purification of secreted PLF will be described elsewhere). The immunologically purified protein was reduced and subjected to sequential Edman degradation (17) with the result shown in Fig. 5. Secreted PLF begins at Phe^{30}, indicating that cleavage had occurred between Ser^{29} and Phe^{30}, as previously inferred from empirical rules (2).
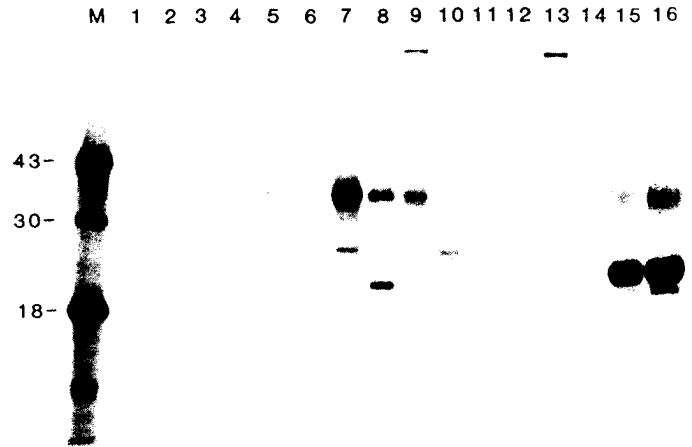

FIG. 4. Secretion of PLF by mouse placenta. Minced placental tissue isolated at 10 (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or 16 (lanes 3, 4, 7, 8, 11, 12, 15, and 16) days gestation was labeled for 2 h with [^{35}S] methionine in the absence (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or presence (lanes 2, 4, 6, 8, 10, 12, 14, and 16) of 5 µg/ml tunicamycin. Supernatants were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with nonimmune (lanes 1–4), anti-PLF (lanes 5–8), anti-mPLI (lanes 9–12), or anti-mPLII (lanes 13–16) serum. M, Marker proteins.
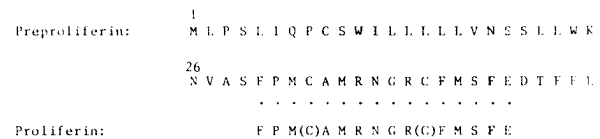

FIG. 5. Comparison of the N-terminal amino acid sequence of PLF inferred from the cDNA sequence and that of secreted PLF determined by sequential Edman degradation. The assignment of the cysteine residues was based on the absence of a peak corresponding to any amino acid.

Discussion

In this communication we described our initial characterization of PLF, a PRL-like protein discovered through cDNA cloning of growth-related mRNAs from BALB/c mouse 3T3 cells (1, 2). PLF cDNA was used to construct a recombinant bacterial plasmid gene, and the resulting bacterial fusion protein was used as an immunogen to raise anti-PLF serum. With this antiserum it was shown that PLF is secreted as a glycoprotein from cells infected with a PLF-SV40 recombinant virus and from BALB/c 3T3 cells, Krebs ascites tumor cells, and mouse placental tissue. The site of cleavage of pre-PLF during secretion was identified by comparing the N-terminal amino acid sequence of PLF purified from the medium of PLF-producing cells with the sequence of pre-PLF inferred from the cDNA sequence. We presume that all, or nearly all, of the glycosylation is N-linked, since in the presence of tunicamycin, secreted PLF has an electrophoretic mobility expected for the protein lacking a signal peptide.

By immunoprecipitation of secreted proteins from

minced placental tissue, we have shown that PLF differs from the midgestational lactogen, mPLI (19) and from the late placental lactogen, mPLII (20), as shown previously (4). Therefore, the placenta appears to secrete at least three immunologically distinct PRL-related proteins. A PLF-related protein identified from a placental cDNA clone by Linzer and Nathans (21) may represent a fourth placental PRL-like protein, although its relationship to mPLI is not yet established (22). PLF does appear, however, to be immunologically related and perhaps identical to mitogen-regulated protein, which was identified by Nilsen-Hamilton and co-workers (23, 24) as a protein secreted by Swiss 3T3 cells in response to stimulation by fibroblast growth factor.

Based on the sequence similarity of PLF and PRL as well as the expression of PLF in rapidly growing cells and placental tissue, we have hypothesized that PLF may be a growth factor for cells in culture and/or for maternal or fetal tissues during pregnancy. Our demonstration that PLF is a secreted protein is consistent with this hypothesis. The availability of purified PLF from the overproducing CHO cell line should allow us to assay for biological and biochemical properties of PLF in an effort to elucidate the possible functions of PLF in cells in culture and in the pregnant mouse.

Acknowledgments

References

1. Linzer DIH, Nathans D 1983 Growth-related changes in specific mRNAs of cultured mouse cells. Proc Natl Acad Sci USA 80:4271
2. Linzer DIH, Nathans D 1984 Nucleotide sequence of a growth-related mRNA encoding a member of the prolactin-growth hormone family. Proc Natl Acad Sci USA 81:4255
3. Linzer DIH, Nathans D 1984 Changes in specific mRNAs following serum stimulation of cultured mouse cells: increase in a prolactin-related mRNA. In: Levine A, Vande Woude G, Topp W, Watson JD (eds) Cancer Cells: The Transformed Phenotype. Cold Spring Harbor Laboratory, Cold Spring Harbor, vol 1:111
4. Linzer DIH, Lee S-J, Ogren L, Talamantes F, Nathans D 1985 Identification of proliferin mRNA and protein in mouse placenta. Proc Natl Acad Sci USA 82:4356
5. Linzer DIH, Talamantes F 1985 Nucleotide sequence of mouse prolactin and growth hormone mRNAs and expression of these mRNAs during pregnancy. J Biol Chem 260:9574
6. Bahl OP, Shah RH 1977 Glycoenzymes and glycohormones. In: Horwitz MI, Pigman W (eds) The Glycoconjugates. Academic Press, New York, vol 1:385
7. Reed R 1983 The resolvase protein of the transposon γδ. Methods Enzymol 100:191
8. Mandel M, Higa A 1970 Calcium dependent bacteriophage DNA infection. J Mol Biol 53:154
9. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680
10. Burnette WN 1981 "Western blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195
11. Brockman WW, Lee TNH, Nathans D 1973 The evolution of new species of viral DNA during serial passage of simian virus 40 at high multiplicity. Virology 54:384
12. Hamilton WG, Ham RG 1977 Clonal growth of Chinese hamster cell lines in protein-free media. In Vitro 13:537
13. Tooze J 1981 DNA Tumor Viruses: Molecular Biology of Tumor Viruses, ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, p 799
14. Pipas JM, Peden KWC, Nathans D 1983 Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T antigen gene. Mol Cell Biol 3:203
15. Sompayrac LM, Danna KJ 1981 Efficient infection of monkey cells with DNA of simian virus 40. Proc Natl Acad Sci USA 78:7575
16. Kahana C, Nathans D 1984 Isolation of cloned cDNA encoding mammalian ornithine decarboxylase. Proc Natl Acad Sci USA 81:3645
17. Hewick RM, Hunkapiller MW, Hood LE, Dreyer WJ 1981 A gas-liquid solid phase peptide and protein sequenator. J Biol Chem 256:7990
18. Struck DK, Lennarz WJ 1977 Evidence for the participation of saccharide-lipids in the synthesis of the oligosaccharide chain of ovalbumin. J Biol Chem 252:1007
19. Soares MJ, Colosi P, Ogren L, Talamantes F 1983 Identification and partial characterization of a lactogen from the midpregnant mouse conceptus. Endocrinology 112:1313
20. Colosi P, Marr G, Lopez J, Haro L, Ogren L, Talamantes F 1982 Isolation, purification, and characterization of mouse placental lactogen. Proc Natl Acad Sci USA 79:771
21. Linzer DIH, Nathans D 1985 A new member of the prolactin-growth hormone gene family expressed in mouse placenta. EMBO J 4:1419
22. Jackson LL, Colosi P, Talamantes F, Linzer DIH, Molecular cloning of mouse placental lactogen cDNA. Proc Natl Acad Sci USA, in press
23. Nilsen-Hamilton M, Shapiro JM, Massoglia SL, Hamilton RT 1980 Selective stimulation by mitogens of incorporation of [³⁵S] methionine into a family of proteins released into the medium by 3T3 cells. Cell 20:19
24. Parfett CLJ, Hamilton RT, Howell BW, Edwards DR, Nilsen-Hamilton M, Denhardt DT 1985 Characterization of a cDNA clone encoding murine mitogen-regulated protein: regulation of mRNA levels in mortal and immortal cell lines. Mol Cell Biol 5:3289