Cell-Free Synthesis of Rat Insulin-like Growth Factor II

ANGELA MARIA ACQUAVIVA, CARMELO BRUNO BRUNI, S. PETER NISSLEY, AND MATTHEW M. RECHLER

SUMMARY

Total RNA extracted from a rat liver cell line (BRL 3A) that synthesizes rat insulin-like growth factor II (IGF-II) was translated in a reticulocyte lysate cell-free system. Incubation of the translation products with antisera to mature rat IGF-II (Mr 8700) selectively immunoprecipitated a Mr 21,600 protein. We propose that this protein represents pre-pro-IGF-II. DIABETES *31*:656–658, July 1982.

Insulin-like growth factor (IGF) II is a Mr 7500 polypeptide isolated from human plasma that is chemically homologous to human proinsulin and to IGF-I.[1] IGF-II resembles IGF-I in biologic activity and receptor reactivity, but differs immunologically. Plasma levels of both IGF-I and IGF-II are low in growth hormone deficiency.[2] Plasma levels of IGF-I, but not of IGF-II, are elevated in growth hormone excess.

Analogous insulin-like growth factors are present in rat plasma. Rat IGF-I[3] appears to be the main postnatal insulin-like growth factor, whereas rat IGF-II is the main insulin-like growth factor in fetal and neonatal serum, suggesting a possible role in fetal development.[4] Growth of pancreatic islet β-cells from newborn rats is potently stimulated by rat IGF-II.[5]

The tissue source of plasma IGFs is not known. Rat IGF-II, also known as multiplication-stimulating activity (MSA), is synthesized by explants of fetal liver,[6] by embryonic fibroblasts in culture,[7] and by the BRL 3A liver cell line.[8] Rat IGF-II from BRL 3A cells has been characterized most extensively. IGF-II polypeptides of Mr 16,300, 8700, and 7100 have been identified in serum-free culture medium after incubation with BRL 3A cells.[9] The three molecular weight forms appear to possess common antigenic determinants.[10] The amino acid sequence of a Mr 7484 species of rat IGF-II is identical to human IGF-II at 62 of 67 loci.[11]

The present study demonstrates that RNA isolated from BRL 3A cells and translated in a rabbit reticulocyte lysate cell-free system directs synthesis of a Mr 21,600 protein that is selectively immunoprecipitated by antisera to Mr 8700 rat IGF-II. We propose that this Mr 21,600 protein may represent pre-pro-IGF-II.

MATERIALS AND METHODS

Materials. Rat IGF-II (Mr 8700) was purified from BRL 3A-conditioned medium as previously described.[9] Antisera no. 422 and no. 2, obtained by immunizing rabbits with rat IGF-II Mr 8700 (Sephadex G-75 MSA, peak II), recognize determinants present on rat IGF-II Mr 8700 and Mr 16,300. Antiserum no. 422, but not antiserum no. 2, also recognizes determinants present on rat IGF-II Mr 7100 (unpublished results).[10]

Guanidine hydrochloride (ultrapure) was purchased from Bethesda Research Laboratories (Bethesda, Maryland); phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), and N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) from Sigma (St. Louis, Missouri); Cowan I strain *Staphylococcus aureus* (Pansorbin) from Calbiochem-Behring (La Jolla, California); and mouse insulin (crystallized, 17.2 U/mg) from Novo Industries (Copenhagen, Denmark).

Extraction of RNA. BRL 3A and BRL 3A2 cell lines[12] were grown to confluence and maintained in serum-free medium for 3–10 days as previously described.[9] RNA was extracted using the guanidine hydrochloride extraction procedure[13] with minor modifications. DNA, protein, and transfer RNA contaminants were removed by ethanol and high-salt precipitations.[13]

Cell-free protein synthesis. Translation studies used rabbit reticulocyte lysate treated with micrococcal nuclease[14] to inactivate endogenous mRNA [New England Nuclear (Boston, Massachusetts), L-[^{35}S]methionine translation kit].

From the Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (A.M.A., C.B.B., and M.M.R.), and the Endocrine Section, Metabolism Branch, National Cancer Institute, National Institutes of Health (S.P.N.), Bethesda, Maryland.

Address reprint requests to Dr. Matthew M. Rechler, National Institutes of Health, Building 4, Rm. B1-14, Bethesda, Maryland 20205.

Received for publication 28 April 1982.

The incubation mixture (25 μl) contained (1) 2–8 μg total RNA; (2) lysate; (3) translation cocktail (spermidine, creatine phosphate, dithiothreitol, and GTP in Hepes buffer); (4) potassium acetate, ~95 mM; (5) magnesium acetate, ~1.4 mM; and (6) L-[^{35}S]methionine, 1215 Ci/mmol, and L-[^{35}S]cysteine, 866 Ci/mmol, 50 μCi (total). Incubation was for 60 min at 37°C.

Immunoprecipitation. Aliquots of translation mixture were incubated with rabbit antisera to MSA, and the antigen-antibody complexes precipitated using the Cowan I strain of *Staphylococcus aureus* containing protein A.[15] Immunoprecipitation buffer contained 0.1 M Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100 (vol/vol); and 50 μg/ml PMSF, TPCK, and TLCK. The translation mixture (50 μl) was preincubated with non-immune rabbit serum (10 μl) and Pansorbin (4 mg, 40 μl) for 30 min at 4°C. Rabbit anti-MSA antiserum (10 μl, 20°C, 1 h) and Pansorbin (8 mg, 40 μl, 4°C, 1 h) were added to the supernatant medium recovered after centrifugation [Beckman microfuge B (Beckman Instruments Inc., Fullerton, California), 4 min]. The immunoprecipitates were collected by centrifugation, washed exhaustively, resuspended in 20 μl of sodium dodecylsulfate (SDS)-gel buffer,[16] boiled for 3 min, and centrifuged. The supernate was stored at −20°C.

SDS-polyacrylamide gel electrophoresis and fluorography. Proteins in the translation mixture or immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis using the discontinuous gel system of Laemmli[16] and a 1.2-mm-thick resolving gel containing 15% acrylamide. Electrophoresis was at constant power (6.5 W). The gel was fixed in 10% trichloroacetic acid (2.5 h), washed twice in water (30 min), incubated in 1 M sodium salicylate (30 min), and dried (Bio-Rad slab gel dryer, Bio-Rad, Inc., Richmond, California). The gels were exposed to Kodak X-Omat R film (Eastman-Kodak, Rochester, New York) at −80°C in a Kodak cassette with regular intensifying screen for 1–15 days.

[^{14}C]-protein standards were myosin, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; cytochrome-C, 12,400 (Bethesda Research Laboratories).

RESULTS

RNA extracted from BRL 3A and BRL 3A2 cells stimulated the incorporation of [^{35}S]methionine and [^{35}S]cysteine into proteins synthesized by a reticulocyte lysate translation system. Trichloroacetic acid-precipitable radioactivity was increased approximately fivefold (not shown). RNA from both cell lines directed synthesis of many proteins with a wide range of molecular weights (Figure 1, panel A).

Incubation of translation products directed by BRL 3A RNA with antiserum (no. 2) raised to rat IGF-II selectively immunoprecipitated a Mr ~22,000 protein (Figure 1, panel B, lane 1, arrow). This protein also was precipitated by a second antiserum (no. 422) to rat IGF-II (not shown), but not by serum from a rabbit not immunized with rat IGF-II (Figure 1, panel B, lanes 5 and 6). The Mr ~22,000 protein also was not observed in immunoprecipitates of translation products directed by RNA from BRL 3A2 cells, a subclone of BRL 3A that does not synthesize and/or secrete rat IGF-II (Figure 1, panel B, lanes 3 and 4),[12] suggesting that BRL 3A2 cells lack active rat IGF-II mRNA.

Immunoprecipitation of the Mr ~22,000 protein was abolished by incubation with excess unlabeled rat IGF-II (Figure 1, panel B, lane 2 and panel C, lane 2). By contrast, unlabeled mouse insulin did not inhibit immunoprecipitation of the Mr ~22,000 protein, despite the fact that rat IGF-II is identical to mouse insulin at 23 of 51 amino acid loci.[11,17]

From several experiments, the apparent molecular weight of this protein was found to be 21,600 ± 1000. The Mr 21,600 protein was labeled by separate incubation with either [^{35}S]cysteine or [^{35}S]methionine (not shown). Immunoprecipitated radioactivity constituted ~0.3% of the trichloroacetic acid-precipitable radioactivity in the translation mixture.

DISCUSSION

RNA isolated from BRL 3A cells directs the in vitro synthesis of a Mr 21,600 protein that is specifically immunoprecipitated by antisera to rat IGF-II. Several lines of evidence suggest that this protein may represent a biosynthetic precursor of the smaller rat IGF-II polypeptides identified in medium conditioned by BRL 3A cells. (1) The Mr 21,600 protein was immunoprecipitated by two antisera raised to Mr 8700 rat IGF-II, but not by nonimmune serum. (2) This protein was not immunoprecipitated from translation products directed by RNA from the BRL 3A2 nonproducer subclone. (3) Immunoprecipitation of the Mr 21,600 protein was competitively inhibited by rat IGF-II, but not by mouse insulin.

Since rat IGF-II is a secreted protein, it is reasonable to presume that the Mr 21,600 protein contains a Mr ~2500

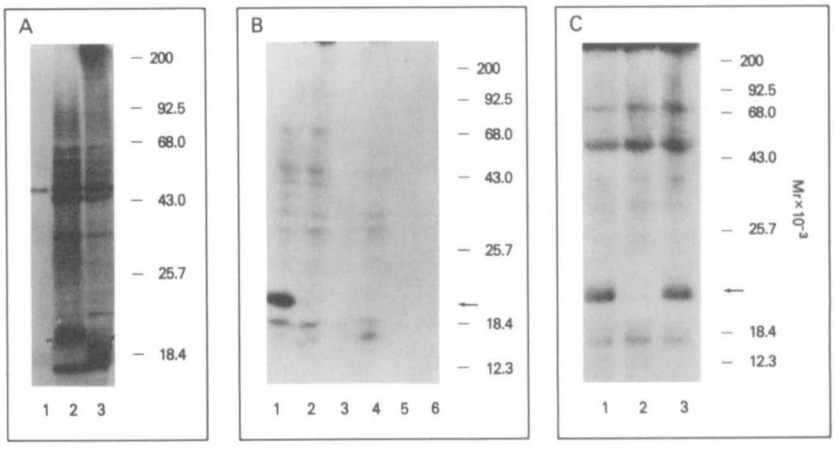

FIGURE 1. Fluorograph of SDS-polyacrylamide gels of [^{35}S]-labeled proteins. Panel A: Aliquots of translation incubations containing no added RNA (lane 1) or 2 μg of RNA from BRL 3A (lane 2) or BRL 3A2 cells (lane 3). Panel B: Translation was directed by BRL 3A RNA (lanes 1, 2, 5, and 6) or BRL 3A2 RNA (lanes 3 and 4). Translation mixtures were immunoprecipitated with antiserum no. 2 (lanes 1–4) or nonimmune serum (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 15 μg of unlabeled rat IGF-II (Mr 8700). Panel C: Immunoprecipitation of BRL 3A RNA-directed translation products by antiserum no. 2 in the absence of unlabeled proteins (lane 1), or in the presence of 15 μg of unlabeled rat IGF-II (lane 2) or 15 μg of unlabeled mouse insulin (lane 3).

prepeptide.[18] The molecular weight remaining for a hypothetical rat pro-IGF-II (19,100) is similar to or slightly larger than that of the largest form of rat IGF-II identified in BRL 3A culture medium (Mr 16,300).[9]

Synthesis of insulin-like growth factors also has been reported from several other cell and organ culture systems. These include explants of fetal rat liver,[6] chick embryo liver,[19] and diverse fetal mouse organs;[20] fibroblasts from human skin[21,22] and rat embryos;[7] and a human fibrosarcoma.[23] In general, these IGF-like peptides have a Mr of 7000–9000. Mr 14,500 and 16,500 forms described in fibrosarcoma[23] and human fibroblast[22] media may represent prohormones or partially processed polypeptides.

Definitive proof that the Mr 21,600 protein represents prepro-rat IGF-II will require demonstration that this molecule contains the sequence of mature rat IGF-II, and that it can be converted to mature rat IGF-II in intact cells or by proteolysis. It is hoped that these studies of IGF-II biosynthesis in BRL 3A cells will provide a model for the regulation of IGF gene expression in normal human and rat tissues.

ACKNOWLEDGMENTS

Dr. Acquaviva was supported in part by a grant to Dr. Matthew M. Rechler from the Kroc Foundation, Santa Ynez, California.

Drs. Acquaviva and Bruni were on leave from Centro di Endocrinologia ed Oncologia Sperimentale del C.N.R. and Istituto di Patologia Generale, IIª Facoltà di Medicina e Chirurgia, Naples, Italy.

REFERENCES

[1] Zapf, J., Rinderknecht, E., Humbel, R. E., and Froesch, E. R.: Nonsuppressible insulin-like activity (NSILA) from human serum: recent accomplishments and their physiologic implications. Metabolism *27*:1803–28, 1978.

[2] Zapf, J., Walter, H., and Froesch, E. R.: Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. J. Clin. Invest. *68*:1321–30, 1981.

[3] Rubin, J. S., Mariz, I., Jacobs, J. W., Daughaday, W. H., and Bradshaw, R. A.: Isolation and partial sequence analysis of rat basic somatomedin. Endocrinology *110*:734–40, 1982.

[4] Moses, A. C., Nissley, S. P., Short, P. A., Rechler, M. M., White, R. M., Knight, A. B., and Higa, O. Z.: Increased levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal rat serum. Proc. Natl. Acad. Sci. USA *77*:3649–53, 1980.

[5] Rabinovitch, A., Quigley, C., Russell, T., Patel, Y., and Mintz, D. H.: Insulin and multiplication stimulating activity (an insulin-like growth factor) stimulate islet β-cell replication in neonatal rat pancreatic monolayer cultures. Diabetes *31*:160–64, 1982.

[6] Rechler, M. M., Eisen, H. J., Higa, O. Z., Nissley, S. P., Moses, A. C., Schilling, E. E., Fennoy, I., Bruni, C. B., Philips, L. S., and Baird, K. L.: Characterization of a somatomedin (insulin-like growth factor) synthesized by fetal rat liver organ cultures. J. Biol. Chem. *254*:7942–50, 1979.

[7] Adams, S. O., Nissley, S. P., Foley, T. P., Greenstein, L. A., Yang, Y. W.-H., and Rechler, M. M.: Multiplication stimulating activity: receptor, growth effects, and production in rat embryo fibroblasts. Program of 63rd Annual Meeting, the Endocrine Society. Cincinnati, Ohio, 1981, p. 238. Abstract No. 621.

[8] Nissley, S. P., and Rechler, M. M.: Multiplication stimulating activity (MSA): a somatomedin-like polypeptide from cultured rat liver cells. Natl. Cancer Inst. Monogr. *48*:167–77, 1978.

[9] Moses, A. C., Nissley, S. P., Short, P. A., Rechler, M. M., and Podskalny, J. M.: Purification and characterization of multiplication-stimulating activity: insulin-like growth factors purified from rat-liver-cell-conditioned medium. Eur. J. Biochem. *103*:387–400, 1980.

[10] Moses, A. C., Nissley, S. P., Short, P. A., and Rechler, M. M.: Immunological cross-reactivity of multiplication stimulating activity polypeptides. Eur. J. Biochem. *103*:401–408, 1980.

[11] Marquardt, H., Todaro, G. J., Henderson, L. E., and Oroszlan, S.: Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. J. Biol. Chem. *256*:6859–65, 1981.

[12] Nissley, S. P., Short, P. A., Rechler, M. M., Podskalny, J. M., and Coon, H. G.: Proliferation of Buffalo rat liver cells in serum-free medium does not depend upon multiplication stimulating activity (MSA). Cell *11*:441–46, 1977.

[13] Paterson, B. M., and Roberts, B. E.: Structural gene identification utilizing eukaryotic cell-free translational systems. *In* Gene Amplification and Analysis. Vol. 2. Chirikjian, J. G., Ed. Holland, Elsevier-North, 1981, pp. 417–37.

[14] Pelham, H. R. B., and Jackson, R. J.: An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. *67*:247–56, 1976.

[15] Kessler, S. W.: Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. *115*:1617–24, 1975.

[16] Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*:680–85, 1970.

[17] Humbel, R. E., Bosshard, H. R., and Zahn, H.: Chemistry of insulin. *In* Handbook of Physiology. Vol. I, section 7. Steiner, D. F., and Freinkel, N., Eds. Washington, D. C., American Physiological Society, 1972, pp. 111–32.

[18] Blobel, G., and Dobberstein, B.: Transfer of proteins across membranes. J. Cell Biol. 67:835–51, 1975.

[19] Haselbacher, G. K., Andres, R. Y., and Humbel, R. E.: Evidence for the synthesis of a somatomedin similar to insulin-like growth factor I by chick embryo liver cells. Eur. J. Biochem. *111*:245–50, 1980.

[20] D'Ercole, A. J., Applewhite, G. T., and Underwood, L. E.: Evidence that somatomedin is synthesized by multiple tissues in the fetus. Dev. Biol. *75*:315–28, 1980.

[21] Clemmons, D. R., Underwood, L. E., and Van Wyk, J. J.: Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. J. Clin. Invest. 67:10–19, 1981.

[22] Atkison, P. R., and Bala, R. M.: Partial characterization of a mitogenic factor with somatomedin-like activity produced by cultured WI-38 human fibroblasts. J. Cell Physiol. *107*:317–27, 1981.

[23] Marquardt, H., Wilson, G. L., and Todaro, G. J.: Isolation and characterization of multiplication-stimulating activity (MSA)-like polypeptide produced by a human fibrosarcoma cell line. J. Biol. Chem. *255*:9177–81, 1980.