Biosynthesis of Islet Amyloid Polypeptide

ELEVATED EXPRESSION IN MOUSE βTC3 CELLS*

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Islet amyloid polypeptide (IAPP) messenger RNA levels, biosynthesis, processing, and secretion were studied in cultured mouse βTC3 insulinoma cells. Northern blot analysis revealed that the size of IAPP mRNA (0.9 kb) in βTC3 cells was the same as that in normal mouse islets; IAPP mRNA was approximately 60% of the level of insulin mRNA in βTC3 cells. However, the ratio of synthesis of insulin to IAPP was approximately 6:1, suggesting that IAPP mRNA is not translated efficiently in these cells. Metabolic labeling of βTC3 cells with [3H]leucine revealed the synthesis of both a precursor form of IAPP (pro-IAPP) of apparent M, 7400 and a mature form (IAPP) of apparent M, 3900. In pulse-chase experiments, pro-IAPP could be shown to be processed to IAPP in a manner similar to proinsulin. The t1/2 for conversion of pro-IAPP to IAPP was about 25 min, faster than the t1/2 for proinsulin to insulin of 70 min. A significant proportion of newly synthesized IAPP and insulin precursors were secreted via a constitutive pathway from βTC3 cells. Possible effects of dexamethasone and forskolin on IAPP mRNA levels and biosynthesis were examined but no effects were observed. In conclusion, the IAPP gene is strongly expressed in βTC3 cells leading to the biosynthesis, proteolytic processing, and secretion of IAPP, a putative islet hormone.

Amyloid deposits are found in the pancreas of >90% of NIDDM patients and are also present in >50% of insulin-producing tumors (1–3). Although the insolubility and low concentration of islet amyloid hampered its biochemical analysis, the recent isolation and sequence analysis of this material revealed that a major component of the deposits is a peptide, termed amyloid polypeptide (IAPP), which consists of 37 amino acids and is structurally related to calcitonin gene-related peptide (4).

Recently, several laboratories have succeeded in isolating cDNAs encoding human and/or rat IAPP precursors (5–7). These studies have shown that prepro-IAPP has a typical signal peptide followed by a relatively short prohormone-like sequence which contains the IAPP sequence in its central region. IAPP has been shown to be present in normal pancreatic islets in measurable amounts (8) and has been localized by immunocytochemistry to the secretory granules of the B cells in normal adult islets (9–12). Thus, it is postulated that IAPP is synthesized as a prepropeptide in islet B cells and then processed proteolytically at diabasic residues in the early secretory granules, in a manner similar to that for proinsulin. However, the low content of IAPP in normal rat islets (13) requires that studies of its biosynthesis be carried out by immune precipitation with specific antisera.

In the present study, we found that mouse βTC3 cells, a clonal cell line derived from SV40 T antigen-induced insulinoma in transgenic mice (14), express high levels of IAPP mRNA and synthesize easily detectable levels of IAPP, which has enabled us to study its biosynthesis, processing and secretion in considerable detail.

EXPERIMENTAL PROCEDURES

Materials—L-[4,5-3H]Leucine (145 Ci/mmol), Amplify™, and a kit for nick translation were from Amersham Corp. [α-32P]dCTP was purchased from Du Pont-New England Nuclear. Protein A-Sepharose was from Pierce Chemical Co. Nitrocellulose membranes were from Schleicher & Schuell. Dexamethasone and forskolin were purchased from Sigma.

Cell Culture—The βTC3 cell line was kindly provided by D. Hanahan (University of California at San Francisco, CA), and grown at 37°C with Dulbecco’s minimal essential medium supplemented to a final glucose concentration of 4 mg/ml containing 10% fetal bovine serum, 25 mM Hepes, 1 mM L-glutamine, minimal essential medium-nondependent amino acids, and 0.5 mg/ml gentamicin.

Labeling of Cells—After cells were incubated in RPMI 1640 medium (v/v 10% fetal bovine serum) under the conditions described in each figure legend, 106 cells in 1.0 ml were pulsed with [3H]leucine (300 μCi/ml) in leucine-deficient RPMI 1640 medium, as described in each figure legend. For chase analyses, the pulse-labeled cells were washed twice in complete medium and incubated in complete medium appropriate time intervals.

IAPP Biosynthesis—Cells were lysed by the freeze and thaw method, lyophilized, and dissolved in 0.5 ml of TASS buffer (0.1 M Tris-HCl, 0.05 M NaCl, 0.25% bovine serum albumin, pH 7.6) containing protease inhibitors (5 × 10–7 M phenylmethylsulfonyl fluoride, 50 μg/ml pepstatin, 50 μg/ml trysyl, 1 μg/ml leupeptin, and 5 × 10–4 M EP-459). The cell lysate and supernatant after the chase incubation were immunoprecipitated in a volume of 0.4 ml with 1 μl of rabbit anti-rat IAPP antisera (Peninsula Laboratories) and pelleted with Protein A-Sepharose after prior absorption with normal rabbit serum. The immunoprecipitates were washed once in 0.6 M NaCl, 10 mM Tris (pH 8.6), 0.05% Nonidet P-40, and 0.1% SDS before electrophoresis on 17.5% polyacrylamide slab gels according to Laemmli (15) with minor modifications. The gels were then treated with Amplify™ and dried, and the radioactivity was visualized by autoradiography after 7 days in ~80°C.

Insulin Biosynthesis—Immunoprecipitation of insulin and proinsulin was performed with guinea pig anti-insulin antisera as described elsewhere (16). Proinsulin and insulin were separated on 1–5% Bio-Gel P–30 column in 3 M acetic acid as described elsewhere (17).

RNA Analysis—Total cellular RNA was prepared from βTC3 cells by the guanidine isothiocyanate procedure (18). RNA was analyzed by electrophoresis through 1.5% agarose, 0.66 M formaldehyde gel,
RESULTS AND DISCUSSION

IAPP Gene Expression—To examine the level of IAPP expression in βTC3 cells total RNA was extracted from both freshly isolated mouse islets and βTC3 cells maintained in DMEM containing 22 mM glucose for 24 h, and subjected to Northern analysis. As shown in Fig. 1, the 0.9-kb IAPP transcript in βTC3 cells was the same size as that in normal mouse islets. However, the ratio of IAPP to insulin mRNA in βTC3 cells appeared to be relatively higher than in normal mouse islets. To more accurately estimate this ratio in βTC3 cells or mouse islets equal amounts of mouse IAPP and insulin cDNAs were nick-translated together to obtain probes having similar specific activities. When 50-pg–1-ng amounts of both unlabeled mouse IAPP and insulin cDNAs in the cloned plasmid PGEM 4Z were dot-blotted as standards (Fig. 2), the hybridization signal with insulin cDNA was approximately 2-fold stronger than that with IAPP cDNA based on densitometric analysis. Northern analysis (also shown in Fig. 2) revealed that IAPP mRNA (0.9 kb) was adequately separated from insulin mRNA (0.6 kb) in a 1.5% agarose/ formaldehyde gel. The estimated concentration of IAPP mRNA in βTC3 cells was about 60% that of insulin mRNA based on the standards, while in mouse islets, the content of IAPP mRNA was approximately 20% that of insulin mRNA. Thus, the βTC3 cell line produced a much higher level of IAPP mRNA compared with that in normal mouse islets.

Biosynthesis of IAPP—Mouse βTC3 cells were labeled with [3H]leucine for 3 h in either 0 or 11 mM glucose medium after 48-h preincubation of cells in RPMI 1640 medium containing either 0 or 11 mM glucose, respectively. Radioautography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of proteins immunoprecipitated from labeled cell extracts with a rabbit anti-rat IAPP antiserum revealed two major bands (Fig. 3). These proteins were identified as pro-IAPP and mature IAPP on the basis of the following criteria: 1) they migrated as 7.4-kDa and 3.9-kDa proteins, respectively, as expected on the basis of their predicted Mz; 2) they were not immunoprecipitated with a nonimmune control serum; 3) the addition of 10 μg of unlabeled rat IAPP displaced both bands (see Fig. 7); 4) the 3.9-kDa band comigrated with iodinated rat IAPP (data not shown); and 5) the 7.4-kDa band became labeled earlier in pulse chase experiments (see next section).

The lack of any effect of glucose on IAPP biosynthesis may be due to the high passage level of this cell line. In earlier experiments we found that the synthesis of insulin and tri- chloroacetic acid-extractable total protein in this cell line was regulated by glucose, but this responsiveness was lost during progressive passages of the cell line. In a parallel experiment, the synthesis of both IAPP and insulin was measured as shown in Table I. Interestingly, the rate of insulin synthesis was about 6-fold higher than that of IAPP, despite the finding (Fig. 2) that the mRNA level for insulin is only about 1.7-fold higher than that for IAPP.

2 S. Nagamatsu, M. Nishi, and D. F. Steiner, manuscript in preparation.

Fig. 1. Northern blot analysis of IAPP and insulin mRNA from both mouse islets and mouse βTC3 cells. Total cellular RNAs from both mouse islets isolated by collagenase digestion as described previously (34) and mouse βTC3 cells were isolated as described under "Experimental Procedures." Lane A, Northern analysis of total cellular RNA (10 μg/lane) probed with a nick-translated [α-32P]dCTP-labeled mouse IAPP cDNA; lane B, rehybridization of the Northern blot used in A with a nick-translated mouse insulin cDNA. The film was exposed for 4 h at -80°C with intensifying screens.

Fig. 2. Quantitation of IAPP and insulin mRNAs in βTC3 cells or mouse islets by a blot hybridization assay using cloned complementary DNAs as standards. A, as the standards, mouse IAPP and insulin cDNAs were denatured at 70°C for 15 min and applied to nitrocellulose under a weak vacuum. The blot was hybridized with a nick-translated probe, which was prepared using equal amounts of both mouse IAPP and insulin cDNAs in the same tube. Lanes: 1, 50 pg; 2, 100 pg; 3, 250 pg; 4, 500 pg; 5, 1 ng. B, Northern blot analysis of total cellular RNA from βTC3 cells (lane 1, 10 μg; lane 2, 15 μg) or normal mouse islets (lane 3, 10 μg). The blot was hybridized with the probes described above. The film was exposed for 2 h at -80°C.

Fig. 3. Identification of pro- and mature IAPP-related protein bands. βTC3 cells were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of either 0 or 11 mM glucose for 48 h, and then pulse labeled for 3 h with [3H]leucine under the same conditions. Cells were lysed and immunoprecipitated with rabbit anti-rat IAPP serum as described under "Experimental Procedures." The immunoprecipitates were analyzed on 17.5% SDS-PAGE after fluorography with Amplify™ and autoradiography for 7 days at -80°C.

Biosynthesis of Islet Amyloid Polypeptide

The polymerase chain reaction using specific oligonucleotide primers based on the published sequences (19, 20). Filters were washed under high stringency conditions (0.1 × SSC, 0.1% sodium dodecyl sulfate, 65°C) and autoradiographed with intensifying screens.
pro-IAPP to converting enzyme in the secretory granules is not known. The reasons for this difference are not known. Since the biosynthesis of IAPP was only 17% lower than that of proinsulin, which may allow for more rapid conversion of pro-IAPP. On the other hand, it is probable that pro-IAPP is processed preferentially by one of the putative prohormone processing enzymes in the early secretory granules (presumably the relatively Lys-Arg-specific Type II enzyme (21)) while the other (Type I) may be rate limiting for proinsulin processing.

As shown in Fig. 4, only pro-IAPP was found in the medium after either 30 or 60 min of chase incubation. Since the conversion rate of IAPP is rapid and IAPP is cosecreted along with insulin (22, 23), mature IAPP must be retained in secretory granules for at least 60 min during the chase, prior to their regulated release. However, Fig. 6 shows that up to 60% of the total labeled pool of either proinsulin or pro-IAPP was secreted from those cells during the 90-min pulse-chase period, while only about 2% of the total proinsulin was secreted from normal mouse islets during a 2-h chase (24). These data suggest that a significant fraction of both newly synthesized proinsulin and pro-IAPP is secreted via an unregulated, or constitutive, pathway, as is also the case in the pituitary corticotroph AtT20 cell line transfected with a human preproinsulin cDNA (25), as well as isolated islets from transgenic mice expressing an Asp B10 mutant human proinsulin gene (24).

**Effects of Dexamethasone and Forskolin on Gene Expression and Biosynthesis of IAPP**—Glucocorticoids alter transcriptional rates of specific genes and have been reported to bind tRNA species required for general protein synthesis (26). Glucocorticoids have been shown to increase pancreatic islet insulin mRNA content (27-29). However, in the HIT cell line, dexamethasone inhibits proinsulin biosynthesis (30).
measured, as described earlier. However, 1 μM dexamethasone and 20 μM forskolin failed to affect these parameters (Fig. 7). Proinsulin biosynthesis also was not affected by either dexamethasone or forskolin in βTC3 cells (data not shown).

In summary, we have studied IAPP mRNA levels and the biosynthesis, processing, and secretion of IAPP in mouse βTC3 cells. These transformed β cells exhibit a number of significant differences from normal islet β cells. The level of expression of IAPP mRNA is considerably higher than in normal β cells which is consistent with observations on various clonal derivatives of a rat insulinoma which are heterogeneous with respect to insulin and IAPP expression (35). Taken all together these findings indicate that the expression of IAPP and insulin are not tightly linked and thus some insulinomas may express unusually high or low levels of IAPP. Although βTC3 cells expressed abundant IAPP mRNA, the biosynthesis of IAPP was lower than for insulin, probably due to less efficient translation of the IAPP mRNA. The t1/2 of conversion of pro-IAPP to mature IAPP was about 25 min. Newly synthesized pro-IAPP was partly secreted via a constitutive pathway in βTC3 cells, unlike normal β cells which release almost no prohormone via unregulated pathways. Finally, no effects of dexamethasone or forskolin were observed on either IAPP gene expression or biosynthesis. Thus, βTC3, like other transformed neuroendocrine cells differ in several differentiated properties including levels of hormone expression, efficiency of prohormone sorting and regulation of both translation and secretion.

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Biosynthesis of Islet Amyloid Polypeptide

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