IDENTIFICATION OF INSULIN SYNTHESIZING POLYSOMES IN ISOLATED ISLETS OF LANGERHANS FROM RAT PANCREAS

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1. Introduction

The use of immunological methods for binding nascent peptide is a promising tool for the detection of polysomes specifically involved in the synthesis of certain proteins. It has been shown that antibodies against completed protein chains may react with the corresponding nascent polypeptides on the polysomes [1–4]. We were interested to characterize the polysomes active in proinsulin biosynthesis because this might make it possible to isolate proinsulin specific messenger-RNA. Since the islets of Langerhans are embedded in the large reservoir of ribonuclease-rich acinar structures of the exocrine pancreas, a major problem is to protect the islet polysomes against the attack of ribonuclease. This difficulty may be overcome by the use of crystalline heparin or other ribonuclease inhibitors to prevent hydrolytic breakdown. To prevent metabolic breakdown during isolation, the polysomes were 'frozen' by the addition of cycloheximide to the incubation medium [5]. In the present paper we report the isolation of polysomes from islets of Langerhans from rat pancreas and the identification by means of an immunological insulin assay of those polysomes which are active in proinsulin biosynthesis.

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2. Methods

2.1. *Isolation of islets of Langerhans*

The islets of Langerhans were isolated from pancreas of rats weighing 250–300 g with the aid of collagenase (Worthington) according to the technique described by Lacy and Kostianovsky [6] as modified by Gerner et al. [7]. The isolation medium was saturated with carbogen prior to use and contained 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4$, 27 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 3 mM glucose and 200 μg cycloheximide (actidione, Upjohn Co)/ml unless specifically mentioned otherwise. The rats were fed normally except for the addition of glucose to the drinking water (concn. 10%) overnight before sacrifice.

2.2. *Isolation of polysomes*

The islet polysomes were prepared according to the method of Gielkens et al. [8] as follows. About 500–1000 islets were homogenized at 4°C in 0.6 ml polysomal buffer containing 0.05 M Tris–HCl (pH 8.5), 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol, 200 μg cycloheximide/ml and 500 μg heparin/ml and made 0.5% with Nonidet P-40 (Shell Corp.). The homogenate was gently shaken for 5 min after homogenization to obtain complete lysis. The lysate was centrifuged at 800 *g* for 5 min. 0.5 ml of the supernatant was layered directly on an isokinetic sucrose gradient in 0.05 M Tris–HCl (pH 8.0), 0.08 M KCl, 0.005 M magnesium acetate, 200 μg cycloheximide/ml, 500 μg heparin/ml and centrifuged in a SW-41 rotor in a Spinco L2-65B ultracentrifuge at 41 000 rpm for 105 min at 3°C. For isokinetic sucrose

gradients, the mixing vessel contained 12.1 ml 15.9% sucrose and 12.0 ml 39.9% sucrose was run in [9]. The gradients were pumped though a LKB Uvicord to which a Vitatron recorder was attached and monitored for ultraviolet absorption at 254 nm and fractions were collected for radioactivity measurements or immunoassays.

2.3. *Incubations of islets for nascent peptide labelling*

For some experiments the islets were washed 3 times with incubation medium not containing cycloheximide. The incubation medium was the same as the isolation medium except for the further addition of 0.2% bovine serum albumin. 500–1000 islets were then suspended in the homogenizer tube in 0.5 ml incubation medium also containing 17 mM glucose and 2.5 nmoles of each essential amino acid except of L-leucine and pre-incubated for 45 min at 37°C; the preincubation medium was then removed by centrifugation and replaced by 0.5 ml fresh medium, differing from the preincubation medium by the further addition of 5 μCi [^{3}H]L-leucine (52 Ci/mmole, Radiochemical Centre, Amersham). After the islets had been pulse-labelled for 5 min with [^{3}H]L-leucine at 37°C, the incubation was stopped by cooling the tubes in ice water and by adding cycloheximide and heparin at the final concentration of 200 μg/ml and 500 μg/ml respectively. The medium was removed from the islets by centrifugation and the islets were washed twice with and finally suspended in 0.5 ml of the polysomal buffer described in sect. 2.2.

2.4. *Insulin immunoassay*

The gradient fractions were diluted with gradient buffer and centrifuged in a Ti-50 rotor in the Spinco L2-65B ultracentrifuge at 50 000 rpm for 2 hr at 3°C in order to precipitate the polysomes, the ribosomes and the ribosomal subunits. The tubes and pellets were rinsed 3 times with gradient buffer to remove the soluble insulin. The pellets were then suspended in 0.5 ml buffer for the insulin assay. The samples were assayed for insulin immunoreactivity by the double antibody method of Hales and Randle [10] using the insulin immunoassay kit (Radiochemical Centre, Amersham) and rat insulin as the standard.

2.5. *Measurement of radioactivity*

In the studies for nascent peptide labelling the gradient fractions were precipitated with an equal volume of 10% trichloroacetic acid after addition of 250 μg of carrier serum albumin, washed three times with cold 5% trichloroacetic acid, digested in 1 ml Soluene TM 100 (Packard) and counted in liquid toluene-based scintillator.

3. Results and discussion

Preliminary experiments indicated that the isolation of polysomes from islets of Langerhans is not possible unless measures are taken to completely inhibit the pancreatic ribonuclease that is present as a contaminant of the islets. Using the very sensitive method of Mans and Alvarez [11] for measuring the ribonuclease activity by polyacrylamide-gel electrophoresis, we estimated the amount of ribonuclease present in our washed islet preparations. The average contamination was 15 pg per islet. Under the conditions of the experiments to be described it appeared that 500 μg heparin per ml could inhibit pancreatic ribonuclease up to concentrations of 30 ng/ml to about 90%. Heparin has also been used successfully in the isolation of ovalbumin synthesizing polysomes [4]. Other ribonuclease inhibitors were also tested. The inhibitor present in liver supernatant [12], polyvinylsulphate [13] and cytidine-3′-monophosphate [14] were not sufficiently active. Since the isolation of the islets is rather laborious and time-consuming it was found useful to add cycloheximide to the media in all steps of the isolation procedure so as to prevent the further elongation of the nascent peptides [5]. If the polysomes were isolated in the absence of heparin and cycloheximide no real polysome profile was obtained, but the preparations contained mainly free ribosomes and ribosomal subunits and only a small amount of disomes. If, however, the precautions outlined above were taken the polysomal profile showed material up to the region of the pentasomes. Two examples of the profiles thus obtained are shown in figs. 1 and 2.

In order to label the nascent polypeptides, the islets were pulse-labelled with [^{3}H]L-leucine for 5 min as described under Methods. Before giving the pulse the

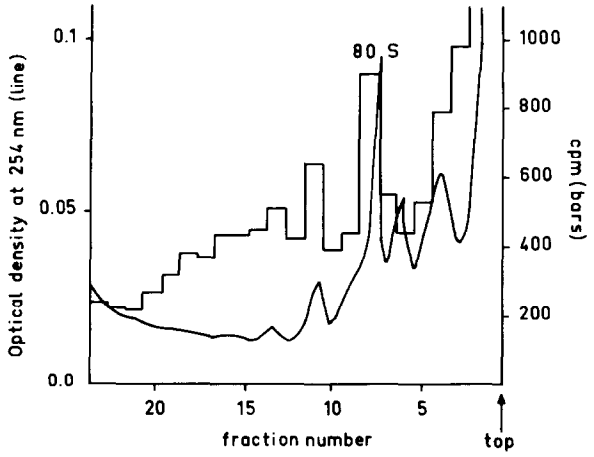

Fig. 1. Sedimentation profile and nascent peptide distribution of polysomes isolated from islets of Langerhans of rat pancreas. 700 islets were incubated at 37°C for 45 min and thereafter pulse-labelled with [^{3}H]L-leucine for 5 min. Polysomes were prepared and analysed as described under Methods. The solid line follows the absorbance at 254 nm: the bars given the radioactivity in the different fractions (cpm). The S-values were calculated using the 70 S ribosome of *Escherichia coli* and the 77 S ribosome of *Neurospora crassa* as the markers. The counting efficiency was 38%.

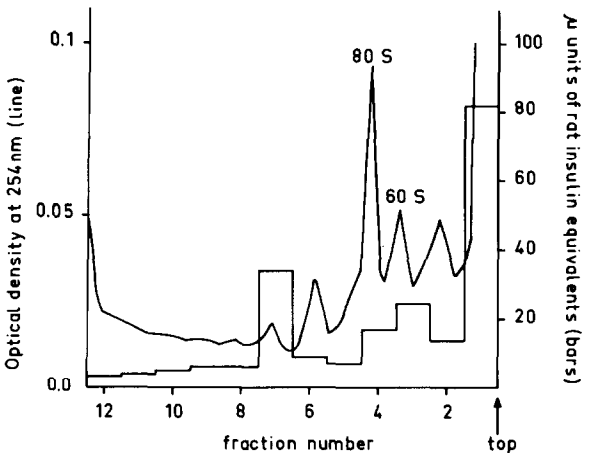

Fig. 2. Sedimentation profile and distribution of 'insulin' immunoreactivity of polysomes isolated from 1000 islets of Langerhans of rat pancreas. All experimental details are given under Methods and in the legend to fig. 1. The solid line follows the absorbance at 254 nm; the bars give the insulin immunoreactivity (μunits of rat insulin equivalents).

islets were washed thoroughly because the cycloheximide added during the isolation had to be removed. A typical radioactivity profile is shown in fig. 1. It can be seen that most of the particles carry nascent peptides. The specific activity in the polysome region of the gradient was much higher than that of the monosomes. The radioactivity in the top of the gradient most likely represents the incorporation into newly synthesized and released polypeptides. Similar radioactivity profiles have been obtained by Permutt and Kipnis [15]. However these authors added liver post-mitochondrial supernatant to their islet preparations as a carrier and to inhibit ribonuclease. The above experiments show that addition of carrier polysomes can be omitted once the necessary precautions for breakdown are taken.

Since it has been demonstrated that proinsulin as such is synthesized as the insulin precursor [16–18], we tried in a subsequent series of experiments to distinguish between the proinsulin synthesizing polysomes and those synthesizing other proteins. As the tool for identification we used the double antibody method [10] for measuring insulin immunoreactivity. Because it is well established that proinsulin gives a cross-reaction with anti-insulin, albeit to a much smaller degree as insulin itself [19], we could rely on the insulin immunoassay kits that are commercially available. The results of a typical experiment are shown in fig. 2. There are 3 peaks of 'insulin' activity. The highest of these is localized at the top of the gradient and has to be ascribed to minute amounts of free insulin still present in the washed pellets of the top fraction(s) of the gradient. It should be mentioned that the original polysome gradient was centrifuged long enough to ensure that the monoribosomes and subunits were migrated sufficiently far from the top as to minimize contamination with free soluble insulin.

The 'insulin' activity was not randomly distributed over the gradient but restricted to the region of the trisomes, notwithstanding the fact that all polysome fractions carry nascent peptides (cf fig. 1). It should be stressed that the insulin equivalents in the polysome fractions are underestimated as compared to the top fraction with at least a factor 3–4 because of the lower reactivity of proinsulin against anti-insulin [19]. The activity in the 60–80 S region has to be attributed most likely to partial breakdown of the polysomes.

Since rat-proinsulin counts 82 amino acids [20], the minimal length of the mRNA for rat proinsulin corresponds with 246 nucleotides, apart from possibly untranslated regions. Assuming that the genetic message for proinsulin is a monocistronic RNA, the finding that the trisomes are preferentially loaded with nascent proinsulin is in good agreement with the general conception that the number of ribosomes in a polysome is roughly linearly related to the length of the message. The latter is evident from the observations of Warner et al. [21] for polysomes synthesizing hemoglobin, of Kuechler and Rich [22] for polysomes active in the synthesis of the light and heavy chains of immunoglobulins and of Vassart and Dumont [23] for polysomes bearing nascent thyroglobulin.

The availability of the methods described here for the preparation of intact polysomes from islets of Langerhans and the selective reactivity with antiserum of those polyribosomes active in proinsulin biosynthesis may be useful in searches for the isolation and characterization of proinsulin mRNA that, in turn, may serve in the unravelling of the mechanism of synthesis and post-translational modification of proinsulin.

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