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MULTIPLE HORMONE SECRETION AND GENE EXPRESSION IN CLONES ISOLATED FROM RAT INSULINOMA CELL LINES

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INDEXING WORDS

RIN; insulin; glucagon; Ha-*ras*; *in situ* hybridization

SYNOPSIS

Current evidence suggests that a multipotential endodermal cell may give rise to all islet cell phenotypes. Five (N1-N5) and twenty-one (m1-m21) pluripotent rat islet cell clones were isolated from two hormone-producing cell line (RIN-r, RINm5F) derived from a radiation-induced islet tumor. To investigate the characteristics of these clones, we analyzed the hormone expression and secretion by Northern blot, immunocytochemistry, and radioimmunoassay. Increased expression and secretion of insulin and glucagon were observed in these clones. The present examination might also be proof of the secretion of both insulin and glucagon in the single cell of the m21 clone isolated from RINm5F. These cell lines also overexpress the Ha-*ras* proto-oncogene. In order to determine whether or not the overexpression was caused by gene translocation, the insulin and Ha-*ras* gene loci in N3 isolated from RIN-r were assigned by in situ hybridization. Both of the genes were located on the long arm of chromosome 1, but no gene translocation was observed. These findings suggest that the expression of insulin and Ha-*ras* is not affected by chromosomal translocation, but it may be functionally linked in these clones. Overexpression of these three genes may indicate that these clones have the same characteristics as the embryonic immature islet cell.

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INTRODUCTION

Biochemical studies of pancreatic β cells have been hampered by the difficulties involved in isolating endocrine tissue from the exocrine pancreas[10]. The transplantable rat islet cell tumor cell lines (RIN-r[7], RINm5F[15]) established in the inbred New England Deaconess Hospital (NEDH) rat have proved to be a valuable source of tissue with which to study various aspects of insulin secretion. These cell lines are capable of continuous growth and are useful in the study of the relationship between the expression of different hormone genes and tumorigenesis. As the loci of both the insulin and Ha-*ras* genes are assigned to chromosome #1 in rat, we believe it is important to determine whether or not these genes affect each other. Whether or not each cell expresses more than one hormone is still unknown[17].

In the present studies, 5 and 21 clones isolated from RIN-r, and RINm5F, respectively; and to determine their multipotentiality, the expression of hormones and proto-oncogenes was analyzed by cloning, Northern blotting, immunocytochemistry, and hormone assay. The karyotype of RIN cell lines was also analyzed to determine any chromosome abnormality.

MATERIALS AND METHODS

Cell culture and cloning

The clones used in this study were isolated from continuous islet cell lines (RIN-r and RINm5F) which had been established from a transplantable rat islet cell tumor[10,15]. Individual cells from the cell lines were cloned by the limiting dilution method. The isolated clones were maintained in RPMI 1640 supplement with 10 % heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 2mM Glutamine, 2mM Na-pyruvate, 10mM HEPES, 50U/ml penicillin, and 50μg/ml kanamycin. Incubation was carried out at 37 °C in an atmosphere of 95% air and 5% CO_2.

Chromosome Analysis

Chromosome specimens were prepared as follows: after passage and incubation for 72 hours, the cells were treated with Colcemid at a concentration of 0.01-0.02mg/ml for the final 1 hour of culture. After harvesting, the cells were subjected to hypotonic treatment with 0.55% KCl solution (30 minutes), and fixed with 3:1 methanol-glacial acetic acid solution only. Cell suspensions were dropped onto slides

and kept in a desiccator for 1-2 weeks. Metaphases were stained with Giemsa after trypsinization.

Probes

The plasmid pBR322 containing the HindIII 0.44kb rat insulin c-DNA specific fragment was kindly supplied by Dr. Rutter W.J. (UCSF). The plasmid pBR322 containing the PSTI 1.2 kb bovine glucagon cDNA specific fragment was kindly supplied by Lopez L.C. via the Japanese Cancer Research Resources Bank, and the plasmid pHas-64sII containing the HindII/PstI 0.68kb v-Ha-*ras* specific fragment was kindly supplied by Dr. Witte O.N. (UCLA).

RNA extraction and Northern blot

Total RNA was extracted by the guanidine thiocyanate (GTC) method[5], transferred onto nylon membranes (Biodyne A, Nihon Pall, Tokyo, Japan) by the method of Thomas[28], and hybridized with probe DNA labeled with [^{32}P]dCTP (cytosine triphosphate) by nick translation (N5000, Amersham, Buckinghamshire, UK).

In situ hybridization

The in situ hybridization procedure was essentially based on the method described by Harper and Saunders[8]. Slides were incubated at 37°C for 1 hour in RNase(Sigma, ST. Louis, USA) at 100μg/ml to eliminate endogenous RNA, rinsed twice in 0.3M NaCl/0.03M sodium citrate (pH 7.0) and a graded series of ethanol and then air-dried. Chromosome DNA was denatured by using 70% (vol/vol) formamide in 0.3M NaCl/0.03M sodium citrate (pH 7.0) at 70°C for 2 minutes and then immediately chilled in ice-cooled 15mM NaCl/ 1.5mM sodium citrate. The slides were then rinsed again in a graded series of ethanol and air dried. Hybridization was performed by incubation in a humid chamber at 43°C for 14-18 hours in ^{3}H-labeled probe DNA, at a concentration of 200ng/ml in 50% (vol/vol) formamide, 0.3M NaCl/0.03M sodium citrate, 0.05M sodium phosphate (pH 6.5), 10% dextran sulfate (Pharmacia, Uppsala, Sweden), and 250 μg/ml of sonicated, denatured salmon sperm DNA. Approximately 40 μl of this mixture was placed onto each slide and sealed with a coverslip. After hybridization, the coverslips were removed in 0.3M NaCl/0.03M sodium citrate/50% formamide and the slides were washed in 0.3M NaCl/0.03M sodium citrate five times within several hours then rinsed in a graded series of ethanol and air-dried.

Autoradiography

The slides were dipped into an aqueous dilution (1:1, vol:vol) of Konica-NRM-2 autoradiographic emulsion (Konica, Tokyo, Japan), air-dried, placed in dark boxes containing a desiccant, and kept at 4°C for 2 weeks. Slides were developed with Konidol-X developer (Konica, Tokyo, Japan) at 20°C for 4 minutes, rinsed in distilled water for 1 minute, fixed with F5 fixer at 4°C for 5 minutes, rinsed in distilled water for 10 minutes, and stained with 10% Giemsa solution. The metaphase plates photographed before hybridization were identified and photographed again.

Immunocytochemistry

Cells were cultured on the glass slides for 3 days. Each slide was washed with PBS twice, and immediately fixed by microwave irradiation according to the method described elsewhere[12,13]. Briefly, the slides were soaked in diluted aldehyde solution (2% [vol/vol] formaldehyde, 0.05% [vol/vol] glutaraldehyde, 0.05 mol/L cacodylate buffer, and 0.025% [weight/vol] calcium chloride (pH 7.35) and irradiated with 2,450 MHz microwave energy at 500 watts for about 20 seconds in formalin and in glutaraldehyde for 15 seconds. After irradiation, the slides were washed briefly in distilled water, and stored at 4°C. Insulin, glucagon, and somatostatin polyclonal antibodies (Dako Japan, Kyoto, Japan) were used for the immunocytologic staining performed by the peroxidase-antiperoxidase (PAP) technique[23].

RESULTS

Morphology of the cell lines

RIN(RIN-r and RINm5F) grew in monolayers with well-defined margins with some of them demonstrating cytoplasmic processes. Despite their common origin, morphologic features between these cell lines were different.

Chromosome analysis

RIN-r had a hypodiploid chromosome with a modal number 40 (Fig. 1), and RINm5F had a nearly hypotetraploid chromosome with a widely distributed modal number. RIN-r had marker chromosomes 1q+ and 3q+.

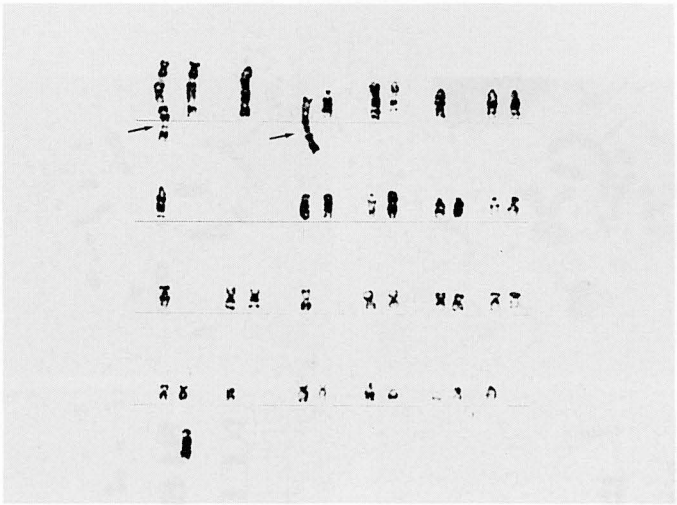

Fig. 1; G-banded karyotype of RIN-r(N-3). Arrows indicate marker chromosomes.

In situ hybridization

RIN-r(N3) cells were analyzed for the insulin gene and the Ha-*ras* gene by in situ hybridization (Fig. 2, 3). Among the total of 114 grains in 100 cells, 25 grains accumulated in 1q and 1q+ near the breakpoint. Therefore, Ha-*ras* was assigned to 1q (Fig. 3). Similar results were obtained after hybridizing the rat preproinsulin probe to N3 chromosomes. Among the total of 125 grains in 100 cells, 33 grains accumulated in 1q and 1q+ (Fig. 2).

Hormone gene expression and radioimmunoassay

These cells released both insulin and glucagon into the culture medium. Expression of the genes coding for insulin and glucagon was assessed by Northern analysis. Every clone expressed both insulin and glucagon at various levels (Fig. 4). The translational products of the specific mRNA of RIN-r(N3) and of RINm5F(m13, m21) were measured by radio immunoassay. Results are expressed per 10^6cells/24hr. RIN-r secreted less insulin than RINm5F(m13, m21) (Table 1). The quantity of the hormones paralleled the relative amounts of mRNA detected.

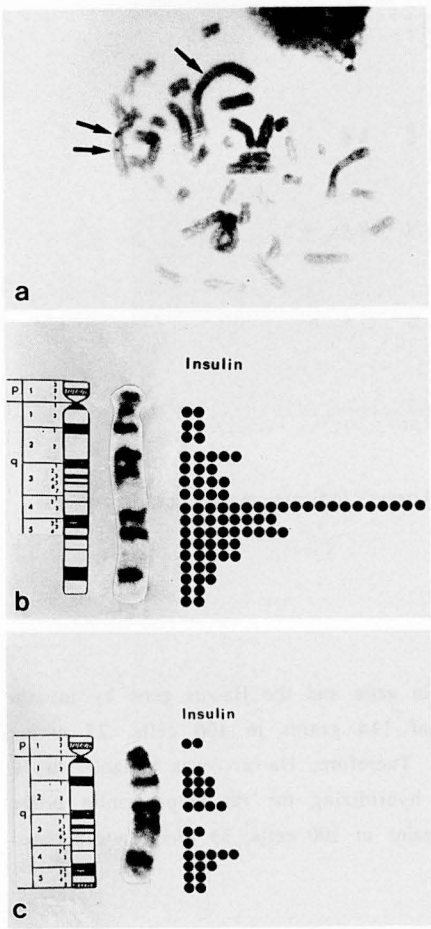

Fig. 2; a) Partial mataphase showing the specific site of hybridization with the Ha-*ras* probe. Arrows indicate silver grains on chromosome 1 and 1q+. b), c) Grain distribution on chromosome 1 and 1q+. Of 114 grains counted, 25 are clustered at 1q or near the break point of 1q+.

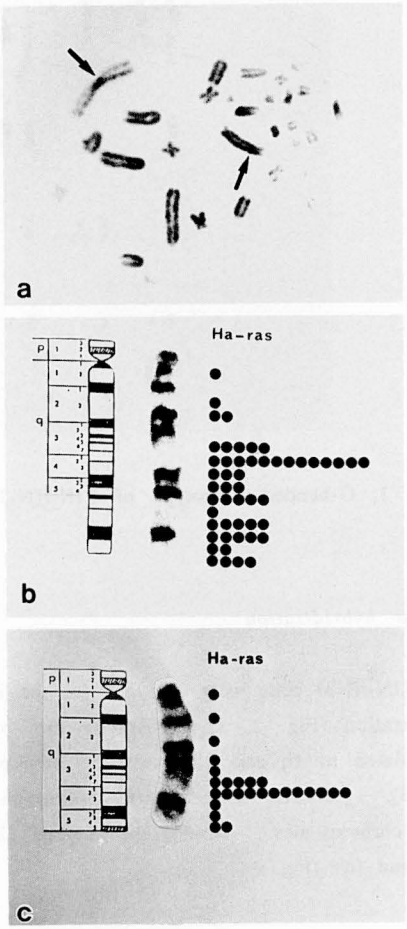

Fig. 3; a) Partial metaphase after in situ hybridization with a preproinsulin probe. Arrows indicate silver grains on chromosome 1 and 1q+. b), c) Distribution of silver grains hybridized with preproinsulin probe on chromosome 1q and 1q+. Of 125 grains counted, 33 are clustered at 1q or near the breakpoint of 1q+.

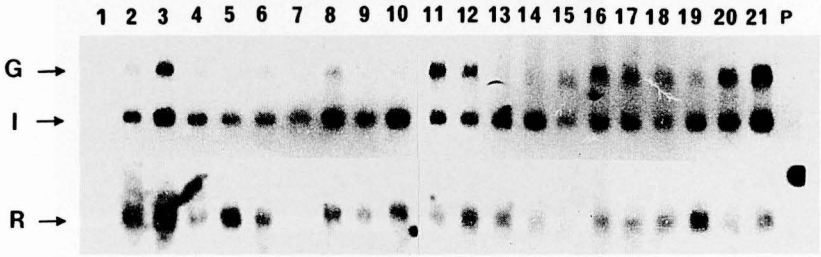

Fig. 4; Northern blot RNA analysis of insulin, glucagon and Ha-*ras*. Total RNA (20μg/lane) extracted from RINm5F(m1-m21, lane 1-21, respectively) underwent agarose gel electrophoresis and capillary transfer to nylon filters. Blots were hybridized with ^{32}P-labeled probes specific to insulin, glucagon and Ha-*ras*. Insulin (I), glucagon (G) and Ha-*ras* (R) mRNA are labeled. Total RNA from rat pancreas (lane P) are analyzed as a control.

Table 1. Hormone secretion of RIN cell lines.

	IRI(μU)*	IRG(pg)*	IRS(pg)*
RIN-r(N3)	1.45	334	<20
RINm5F(m13)	55.2	27.4	<20
RINm5F(m21)	4.71	78.1	<20

* /10^6 cell/24h

IRI: Immunoreactive insulin, IRG: Immunoreactive glucagon

IRS: Immunoreactive somatostatin

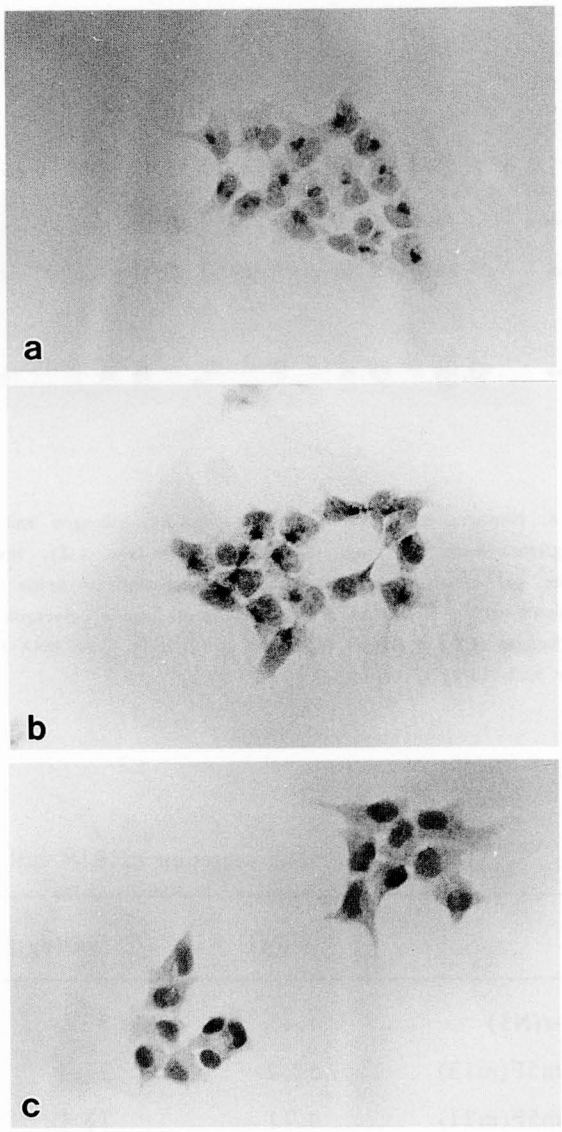

Fig. 5; Immunocytochemistry of RIN cell line(RINm5F, m21). m21 were grown on glass microscope slides and stained with antisera to insulin a), glucagon b), and somatostatin c) described in materials and methods. a)b) In more than 50% cells, amorphous deposits were found near the nuclear membrane. c) No deposit was found in any of the cells. (x 200, original magnification)

Oncogene expression

Expression of genes coding Ha-*ras* and *abl* was assessed by RNA analysis. Every clone expressed more Ha-*ras* than rat pancreas (Fig. 4), but none of them expressed *abl* proto-oncogene. (data not shown)

Immunocytochemistry

Both insulin and glucagon were stained in the cytoplasm of most cells of RIN-r(N3) and RINm5F(m21), but somatostatin was stained in none of these cells (Fig. 5).

DISCUSSION

Rat insulinoma cell lines (RIN-r, RINm5F) have been used as models of pancreatic β cells, and it has been reported that they secret insulin and somatostatin, but that glucagon is not detected in the supernatant fluids.

Our clones secreted and expressed various amounts of insulin and glucagon; however, they secreted neither somatostatin nor pancreatic polypeptide. It has been shown that most of cells derived from RIN do not stain for insulin, glucagon or somatostatin, and that 1-5% of the cells are immunopositive[20]. However, many cells of our clones were stained for insulin and glucagon. These differences may be caused by the method of fixation. It is well known that microwave fixation is one of the better fixation methods in immunohistochemistry. Most of the RIN cells fixed with 10% formalin were not stained for insulin and glucagon. (data not shown) Therefore, detection limits by microwave fixation in immunocytochemistry may be lower compared to the detection limits by the other fixation method. Over 50% of the cells were immunocytochemically positive for both insulin and glucagon, meaning that single cell secreted both insulin and glucagon. This finding suggests that the rat pancreatic islet cell produces a variety of hormones[18].

Karyotype analysis showed that every clone had common marker chromosomes suggesting that these cell lines are derived from a common cell. Recent studies have shown the relationship between islet cell tumors and oncogenes. A new oncogene has been found in the chemical-induced rat islet cell tumor[25]. It has also been reported that glucose stimulates c-*myc* expression and DNA synthesis in RIN[32]. We found the overexpression of Ha-*ras* proto-oncogene in these cell lines. Ha-*ras* serves a fundamental but as yet a poorly defined function in the control of cell growth and development[22]. Stimulation of islet cell mitosis has been induced by the combination of *myc* and *ras*[30]. On the other hand insulin is a very effective

mitogen for RIN cells[6]. These findings suggest that Ha-*ras* and insulin effect the continuous growth of RIN and its isolated clones. It is known that many genes can be induced by the *ras* gene[29]; moreover, insulin which does not induce expression of other growth-regulated proto-oncogenes induces Ha-*ras* mRNA three- to five-fold in quiescent murine fibroblasts[14]. *ras*-encoded protein can mediate insulin-induced maturation of Xenopus oocytes[9]. A recent study has shown that *ras* protein induces a voltage-sensitive inward calcium current[3], and an elevation of cytosolic Ca^{2+} concentration induces insulin secretion in rat islet cell line[31]. These findings support the probability that Ha-*ras* and insulin actions are functionally linked and that Ha-*ras* may induce continuous insulin secretion in RIN.

Oncogene activation is frequently induced by chromosomal translocation[2,4,26]. Because the 1q+ marker is common in these cell lines, and both Ha-*ras*-1 and insulin have been assigned to chromosome #1 of the rat[11,24], the presence of the Ha-*ras* gene or insulin gene at the breakpoint 1q+ had been suspected. In this respect, we performed in situ hybridization by using v-Ha-*ras* and preproinsulin probes and assigned both Ha-*ras* and insulin genes to 1q+ near the breakpoint; however, this translocation did not transpose the Ha-*ras* and insulin from #1 to another chromosome. The enhanced expression of Ha-*ras* and insulin in these cell lines does not seem to have been caused by 1q+ with a rearranged segment. These cell lines have another chromosomal abnormality; 3q+. A previous report has demonstrated that c-*abl* in rat erythroleukemia cell line (K3D) is activated by translocation of chromosome #3[27]. That we could not detect the expression of *abl* in RIN and its isolated clones indicates that translocation of chromosome #3 does not activate c-*abl* in these cell lines.

Current evidence implicates a multipotent endodermal stem cell, capable of differentiation into enterocytes, ducts, acinar, and endocrine cells[19]. Alternatively, the APUD cell (amine precursor uptake and decarboxylation) is postulated as the origin of these cells[16]. Recent studies have suggested the existence of a common ancestor for the peptide-producing cells of the pancreas[1,27], and immature cells containing a few hormones appear throughout prenatal development of mouse pancreas[1].

Our results suggest that RIN and its subclones are immature cells derived from common islet stem cell. Further studies are necessary to differentiate these cell lines into single hormone producing cells.

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