FEBS Letters 425 (1998) 24–28 FEBS 19953

# Differential expression of the protein kinase A regulatory subunit (RIα) in pancreatic endocrine cells

Yoav Arava[a], Konstantin Adamsky[a], Adina Belleli[b], Shmuel Shaltiel[b], Michael D. Walker[a,*]

[a] *Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel*
[b] *Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel*

Received 13 February 1998

**Abstract A PCR-based subtractive cloning procedure was used to identify genes expressed at higher levels in the pancreatic beta cell line βTC1, as compared to the pancreatic alpha cell line αTC1. One of the clones isolated by this procedure corresponded to the regulatory subunit (RIα) of protein kinase A (PKA). Using antibodies directed against RIα, we now demonstrate both by immunoblot and immunofluorescence that RIα protein is present at higher levels in cultured beta cells as compared to alpha cells. In vitro PKA assays revealed high basal PKA activity in αTC1 extracts, which changed little on addition of exogenous cAMP. On the other hand, extracts from beta cells showed very low basal activity of PKA, which was elevated upon addition of cAMP. A similar trend was observed in vivo using transfected luciferase constructs bearing multiple copies of a CRE element: in αTC1 cells, no induction by forskolin was observed, whereas in βTC1 cells, forskolin produced a 9-fold increase in activity. Therefore, the results indicate that RIα of PKA is selectively expressed in pancreatic beta cells as compared to alpha cells: this selective expression is associated with major differences in the properties of the PKA signal transduction pathway. Differential expression of the regulatory subunit may play a role in determining the patterns of gene expression and signal transduction characteristic of alpha and beta cells.**



*Key words:* Alpha cell; Beta cell; Glucagon; Insulin; Transcription; Protein kinase A

## 1. Introduction

The endocrine pancreas contains four distinct cell types, alpha, beta, delta, and PP, which are specialized hormone-secreting cells. Despite the similar morphology and common embryological origins of these cells, they have quite distinct biological functions [1]. The major function of the beta cell is production and secretion of the key metabolic hormone insulin [2], whereas alpha cells selectively express the gene encoding the hormone glucagon [3]. In both cases, secretion is precisely regulated according to metabolic requirements: glucose is a key regulator, exerting direct but inverse effects on beta and alpha cells. Elevated glucose concentrations lead to increased insulin secretion through the activity of glucokinase, a low affinity isozyme of hexokinase, which is expressed in beta cells [4]. High blood glucose levels generate an elevated ATP/ADP ratio, which in turn leads to closure of $K^+$ channels, opening of $Ca^{2+}$ channels and increased secretion of insulin, presumably through phosphorylation of as yet unidentified target proteins [5]. In contrast, elevated glucose levels inhibit glucagon secretion from alpha cells, through mechanisms which may also involve glucokinase, which was recently identified in alpha cells [6].

Most likely, the differences between alpha and beta cells arise during the course of development, as a result of activation of characteristic sets of transcription factors, which in turn lead to expression of alpha- and beta-specific genes. Thus far, only a small number of transcription factors expressed differentially in alpha and beta cells have been identified [7–9]. In order to better understand the distinctive properties of alpha and beta cells, we undertook a subtractive hybridization approach employing representational difference analysis (RDA), a procedure for cloning differentially expressed genes [10]. We chose to compare the established cell lines βTC1 and αTC1, because of the ready availability of mRNA from these cells, and because of the similarity of the procedures used to generate the lines (transgenic mice bearing insulin/glucagon promoter linked to SV40 T antigen [11,12]), which should reduce the number of false positive clones isolated. One of the genes we isolated using this procedure encodes RIα, a regulatory subunit of protein kinase A (PKA), thus confirming a recent report [13].

The PKA signal transduction pathway plays an important role in mediating the effects of a broad range of extracellular signals [14]. Hormonally mediated increases in intracellular cAMP levels lead to dissociation of the catalytically inactive complex of PKA ($R_2C_2$) containing a cAMP-binding regulatory subunit (R) and free (catalytically active) subunit (C); the active catalytic subunit then phosphorylates multiple target proteins including the transcription factor CREB [15]. Four distinct R isoforms (RIα, RIβ, RIIα, RIIβ) and two C isoforms (Cα and Cβ) have been characterized in mouse [16]. The precise role of each of these is not clearly established. There is some degree of functional overlap, since gene disruption of RIβ or RIIβ in mice is accompanied by a compensatory increase in RIα protein [17]. On the other hand, RIα null mutant mice show a phenotype of early embryonic death [17], indicating a unique, non-redundant role for this isoform. Activators of the PKA pathway e.g. forskolin and glucagon-like peptide (GPI) can synergize with glucose in insulin secretion, indicating an important functional link between insulin secretion and the PKA pathway [18,19].

In this report, we demonstrate that the differential expression of RI isoforms at the RNA level is reflected also at the protein level. Furthermore, using in vitro and in vivo assay, we show that this differential expression appears to have important functional consequences on the recruitment of the PKA pathway.

*Corresponding author. Fax: +972 (8) 934 4118.
E-mail: bcwalker@wiccmail.weizmann.ac.il


*PII* S0014-5793(98)00186-0

## 2. Materials and methods

### 2.1. Cell lines

αTC1-6 [20] (mouse glucagon-producing cells) and βTC1 [11] (mouse insulin-producing cells) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% horse serum and 2.5% fetal calf serum and penicillin/streptomycin.

### 2.2. RNA preparation and cDNA-RDA protocol

Total RNA was prepared by using the TRI reagent kit (Molecular Research Center, Inc.) followed by DNAse I treatment for 20 min at 37°C. Poly(A)$^+$ RNA was selected on an oligo dT cellulose column (Boehringer Mannheim). For the cDNA synthesis, two cycles of poly(A)$^+$ selection were performed, and 10 μg of RNA were used to synthesize ds-cDNA [21]. Two μg ds-cDNA were used for the RDA procedure, and all steps were performed as described [10]. The DP3 was cut with *Dpn*II, separated on 1.5% low melting point agarose gel, excised from the gel with Jetsorb resin (Genomed) and cloned into the *Bam*HI site of pBS-KS vector, which was transformed into *E. coli* DH5α or XL-1 blue.

### 2.3. Slot blot analysis

RNA was resuspended in a solution of 50% formamide, 7% formaldehyde, 1×SSC (150 mM NaCl, 15 mM Na citrate) and incubated at 68°C for 15 min. The denatured sample was applied to a Nylon membrane pre-washed with 20×SSC, using a minifold II micro-sample filtration manifold (Schleicher and Schuell), washed twice with 20×SSC and UV cross-linked to the membrane. Probes were prepared by random priming using a hexanucleotide mix (Boehringer Mannheim), and filters were hybridized overnight at 42°C in a hybridization solution containing 50% formamide, 5×Denhardt's solution, 5×SSC, 0.1% SDS and 250 μg/ml denatured salmon sperm DNA.

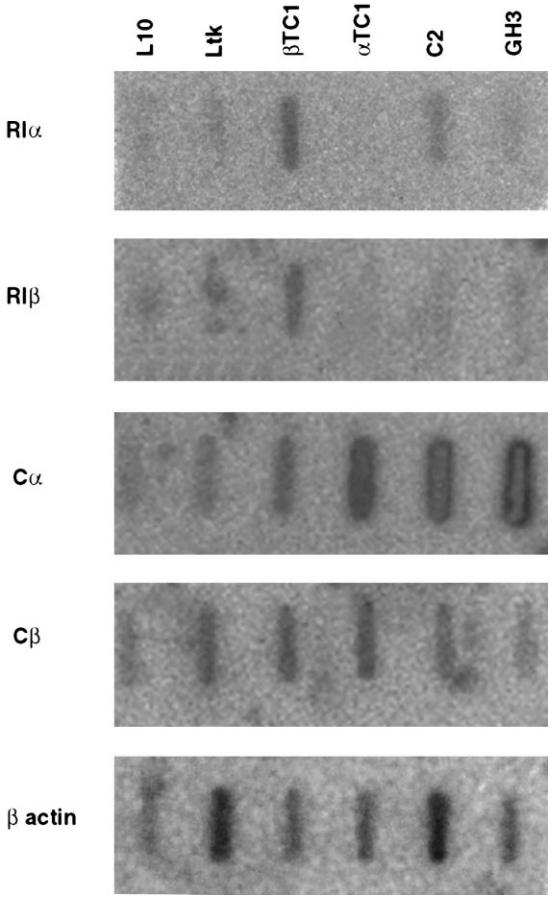


Fig. 1. RNA slot blot analysis: RNA was extracted from the cell lines indicated at the top of the figure and 10 μg of total RNA was applied to a nylon membrane. Membrane strips were hybridized with the probes indicated on the left and exposed to a phosphoimager screen.

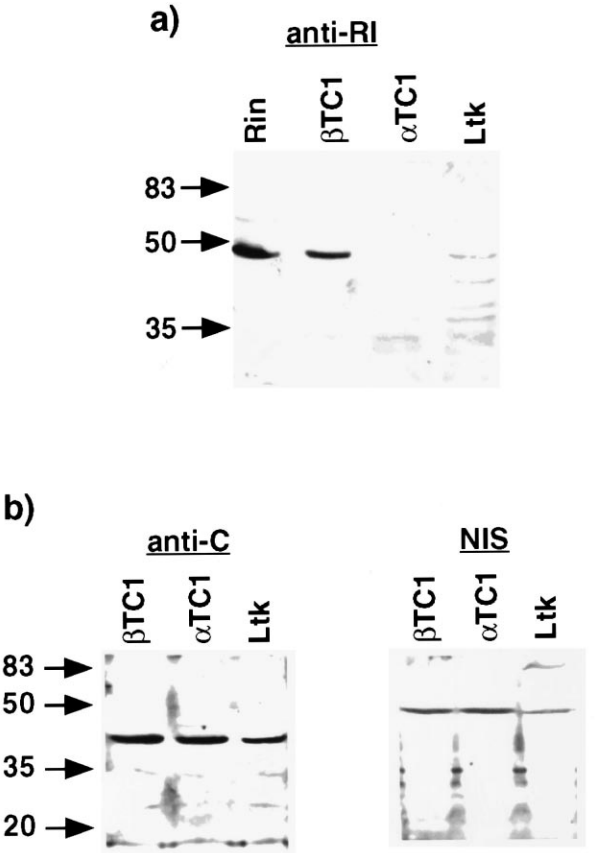


Fig. 2. Immunoblot analysis: total protein was extracted from the indicated cell lines and 100 μg were fractionated on 12% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and incubated with polyclonal antibodies directed against RIα (a), antibodies directed against C or with non-immune sera (NIS) (b). The expected sizes are: RI 48 kDa, and C 40 kDa. The non-specific band detected with NIS does not correspond to these sizes. The migration of molecular weight markers (kDa) is indicated.

### 2.4. Immunoblot analysis

Cells were washed twice with PBS, trypsinized, resuspended in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 50 μg/ml aprotinin) and sonicated. 100 μg protein were separated on 12% SDS-PAGE and transferred to nitrocellulose sheets. Membranes were blocked with 10% (v/v) non-fat milk in 1×PBS and 0.05% Tween-20 for 1 h, followed by incubation for 1 h with primary antibodies, rabbit anti-RIα, or anti-catalytic subunit (antibody alpha P-2) [22] diluted 1:1000 followed by protein A-horseradish peroxidase (1:10 000) for 1 h, and visualization using the ECL (Amersham) reaction.

### 2.5. Immunohistochemical staining

A mixture of βTC1 and αTC1 (1:1 ratio) was plated on a glass cover slip and 48 h later cells were fixed with 4% paraformaldehyde for 20 min, followed by permeabilization with 0.2% Triton X-100 for 5 min. Cover slips were incubated with appropriate concentrations of primary antibodies (rabbit anti-RIα with guinea pig anti-insulin or with guinea pig anti-glucagon) and secondary antibodies (fluorescein-conjugated donkey anti-rabbit and rhodamine-conjugated donkey anti-guinea pig) were incubated for 1 h at room temperature. Cells were examined using a Bio-Rad (model MRC1024) confocal microscope.

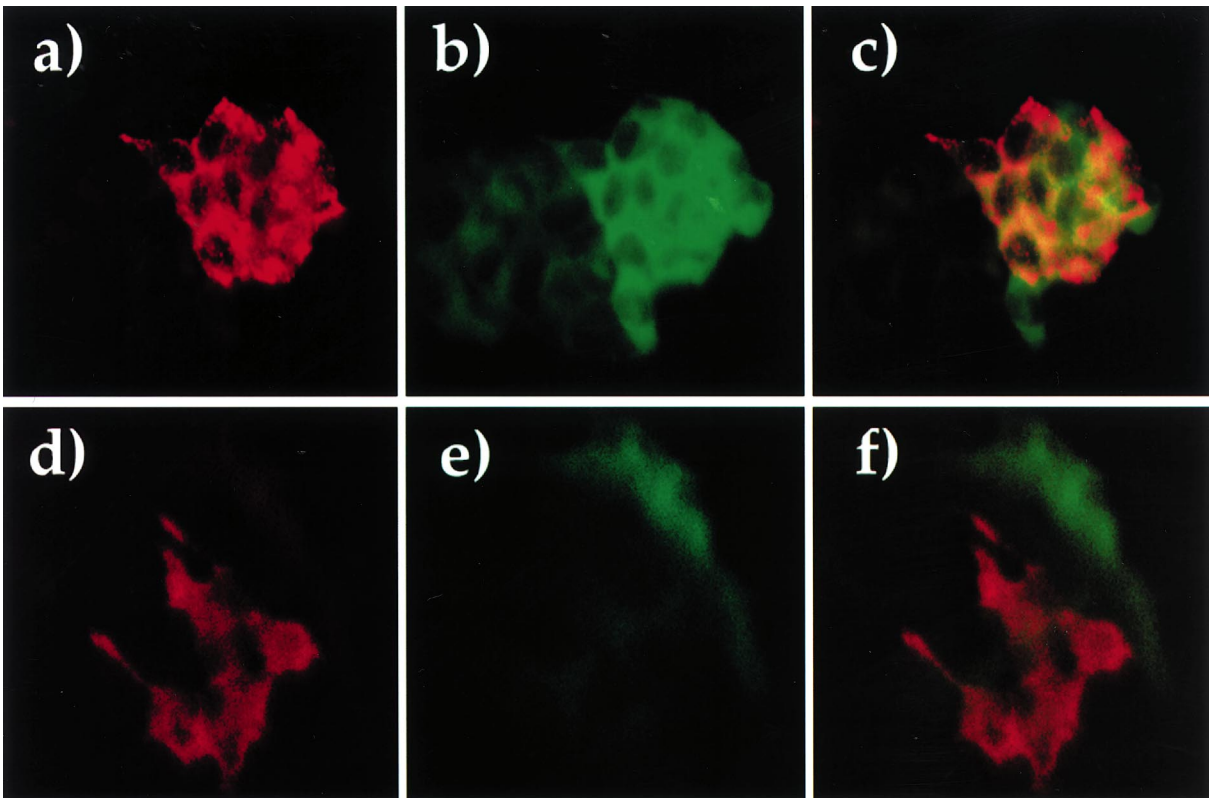


Fig. 3. Immunofluorescent staining of αTC1 and βTC1 cells co-cultured on microscope cover slips. The top panels show a representative single field of cells double stained by incubation with guinea pig anti-insulin and rabbit anti-RIα antibodies followed by rhodamine anti-guinea pig and fluorescein anti-rabbit antibodies and visualization of rhodamine (panel a) fluorescein (panel b) and the combined signals (panel c). The bottom panels show a representative single field of cells double stained by incubation with guinea pig anti-glucagon and rabbit anti-RIα antibodies followed by rhodamine anti-guinea pig and fluorescein anti-rabbit antibodies and visualization or rhodamine (panel d) fluorescein (panel e) and the combined signals (panel f).

*2.6. Kinase assay*

To analyze kinase activity in the protein extracts, extract containing 10 μg protein was incubated for 5 min at 30°C in a reaction containing 50 mM HEPES pH 6.5, 10 mM MgAc, 1 mM EGTA, 100 μM ATP, 10 μM Calmidazolium, 0.25 μCi $\gamma^{32}$P-ATP and 50 μg Histone H2b in a final volume of 50 μl. PKI (Calbiochem) was included, where indicated, at a final concentration of 48 nM. Reactions were spotted on P81 paper, washed 4 times for 15 min with 150 mM phosphoric acid, then once with 70% ETOH and dried. Radioactivity was measured in a liquid scintillation counter using Cerenkov radiation.

*2.7. Transfections*

Cells were transfected with 10 μg of a plasmid encoding a luciferase reporter gene linked to a multimer of the CRE cis-element [23] and 5 μg of the plasmid RSV-CAT as control for transfection efficiency. Glycerol shock (10%) was performed after 5 h, followed by addition of forskolin (10 μM final concentration). Cells were harvested 40 h later and luciferase (Promega luciferase reagent) or CAT assays were performed using the phase extraction procedure [21].

## 3. Results

*3.1. RIα subunit of PKA is selectively expressed in pancreatic β cells*

To compare the expression of the different subunits of PKA at the RNA level, we performed RNA slot blot analysis with RNA from six different mammalian cell lines: lymphoid cells (L10), fibroblasts (Ltk$^-$), pancreatic β cells (βTC1) pancreatic α cells (αTC1), muscle cells (C2), and pituitary endocrine cells (GH3) (Fig. 1). RI mRNA levels (both RIα and RIβ) were highest in βTC1 cells compared to all other cell lines, whereas comparable expression levels were seen for the catalytic subunits Cα and Cβ in the different cell lines. In this experiment, a probe for β actin (a gene expressed in most cell types) was included as a qualitative indicator that comparable amounts of RNA were loaded.

In order to examine expression at the protein level, immunoblot analysis was used. Sera raised against RIα, identified a clear band (corresponding in molecular weight (48 kDa) to the RI subunit) in extracts from βTC1 cells and RIN cells (an independent beta cell line [24]). A much weaker band was seen in Ltk$^-$ fibroblasts, whereas no band was seen for αTC1 cells (Fig. 2a). On the other hand, use of antibodies directed against the catalytic subunit indicated comparable levels of this protein (Fig. 2b).

To confirm this preferential expression of RI subunit in beta cells, we performed immunofluorescence analysis using co-cultured βTC1 and αTC1 cells. Double-labeling using anti-insulin together with anti-RIα antibody (Fig. 3a–c) showed that the insulin-producing βTC1 cells (Fig. 3a, red) correspond with those cells containing high levels of RI (Fig. 3b, green); those cells not producing insulin (presumably αTC1 cells) show much lower levels of RIα (Fig. 3b, green). This is clearly seen in the merged photograph (Fig. 3c) and is confirmed in the reciprocal experiment with anti-glucagon antibodies, showing that the glucagon-producing cells (αTC1) (Fig. 3d) show little or no detectable RI expression (Fig. 3e).

### 3.2. Functional properties of PKA differ between β cells and α cells

To examine the functional consequences of the selective expression of the regulatory subunit we performed kinase assays with protein extracts from both αTC1 and βTC1 cells, in the presence of increasing cAMP concentrations. With αTC1 extracts, high levels of protein kinase activity were seen in the absence of cAMP. This activity is attributable to PKA, since addition of its specific inhibitor, PKI, eliminated essentially all of it (Fig. 4). It should be noted however that addition of increasing concentrations of cAMP, up to $10^{-5}$ M, did not enhance significantly the kinase activity. The complete inactivation of activity by PKI, together with the lack of activation upon addition of cAMP, suggest that in these cells PKA exists in a persistently activated form. In contrast, in βTC1 cells, low levels of activity were detected in the absence of cAMP, increasing 7-fold upon addition of cAMP (Fig. 4). In this case also, PKI was able to efficiently inhibit this activity. This is consistent with the idea that in the absence of cAMP, the catalytic subunit in βTC1 extracts is mainly inactive, presumably as a result of interaction with regulatory subunit. Upon addition of cAMP, the catalytic subunit becomes activated through dissociation of its $R_2C_2$ complex.

The in vivo function of the PKA signal transduction pathway was examined by transfection of cultured cells with plasmids containing a luciferase reporter gene linked to a multimer of the cAMP response element (CRE). Using βTC1 cells, a very low basal level of reporter gene activity was observed; incubation in the presence of forskolin, an adenylate cyclase activator, produced a 9-fold activation (Fig. 5). By contrast, with α cells basal levels were high: addition of forskolin had little or no effect on CRE-dependent transcription (Fig. 5). These results closely parallel the data obtained from in vitro PKA assays. Thus βTC1 cells have a low basal level of activity of PKA, which can be substantially elevated by cAMP. αTC1 cells on the other hand, have a higher basal level, which is not significantly affected by cAMP.

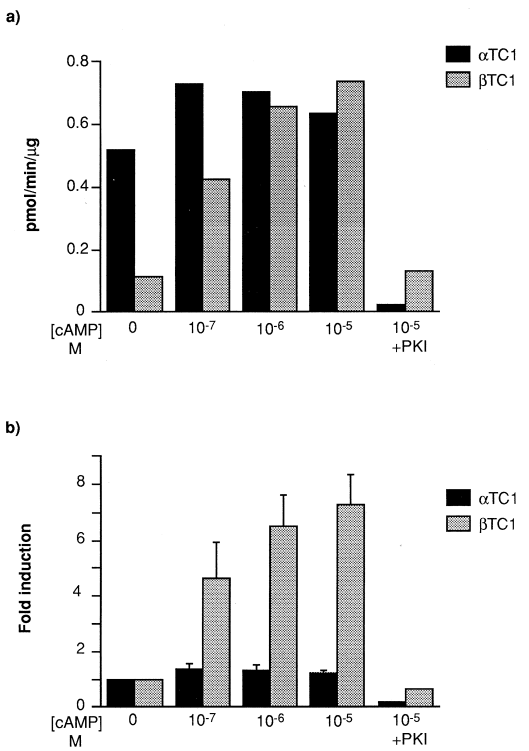


Fig. 4. Kinase activity in αTC1 and βTC1 cells. Phosphorylation reactions were performed as described in Section 2 in the absence or presence of increasing concentrations of cAMP. a: Kinase activity from a representative experiment. b: Fold activation by cAMP (relative to level in the absence of cAMP). Results are the average of three independent experiments ± S.E.M. PKI was added where indicated.

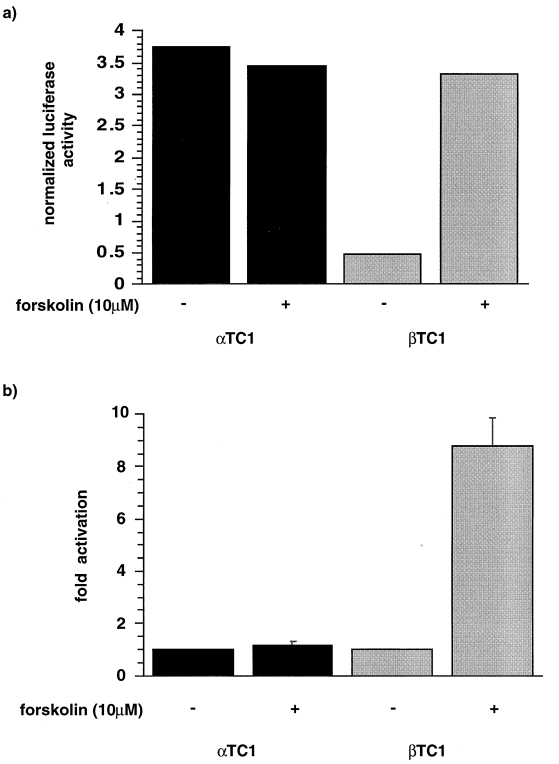


Fig. 5. Effect of forskolin on CRE-dependent luciferase activity in vivo. A reporter plasmid containing 16 copies of the CRE upstream of the luciferase reporter gene, was transfected to αTC1 and βTC1 cells together with the plasmid RSV CAT as internal control. Following transfection, cells were incubated for 40 h in the presence of forskolin. a: Normalized luciferase activity for a representative experiment. b: Fold activation over non-treated cells. Results are the average of three independent experiments ± S.E.M.

## 4. Discussion

Despite similarities in morphology and embryological origins, pancreatic alpha and beta cells have very different physiological roles: glucagon secretion from alpha cells serves to mobilize glucose from the liver and thus elevates blood glucose levels. On the other hand, insulin secretion from beta cells increases glucose uptake by peripheral tissues, thereby decreasing blood glucose levels. In order to accomplish its specialized role of regulated secretion of a unique hormone, each cell expresses a characteristic set of specific genes; presently, relatively few such alpha and beta cell-specific genes are known [13,25]. The current work was undertaken with a view to better understanding the function of alpha and beta cells through cloning of genes expressed differentially between these two cell types. One of the genes thus identified was RIα, a regulatory subunit of PKA.

In this report, we demonstrate that the differential levels of mRNA for RIα lead to substantial differences also at the level

of protein. This is of importance since previous studies have revealed instances of post-transcriptional regulation of R subunit gene expression; for example, disruption of the RIβ or RIIβ genes in mice leads to biochemical compensation by RIα without accompanying changes in RIα mRNA levels [17]. Low levels of RIα protein, as observed in αTC1 cells might be expected to lead to presence of free catalytic subunit and therefore high basal activity of PKA, fully sensitive to PKI inhibition; on the other hand, high levels of RIα protein, as observed in βTC1 cells might be expected to produce low basal, but highly inducible catalytic activity of PKA, again fully sensitive to PKI inhibition. In fact, this is exactly what we have observed upon analysis through both in vivo and in vitro experiments. These results suggest that the RI regulatory subunit plays a dominant role in determining PKA properties in these cells. Consistent with this, we have observed in preliminary immunoblot experiments that levels of RII, unlike RI, are similar in αTC1 and βTC1 cells (data not shown).

What might be the possible physiological implications of differential expression of RI? An interesting indication comes from studies of gene extinction in hybrid cells. Somatic cell hybrids formed between liver cells and fibroblasts display complete inhibition (extinction) of the liver phenotype [26]. It has been shown that this effect is due to expression of a small number of 'extinguisher' loci contributed by the fibroblast parent cell. One of these loci was cloned and shown to correspond to the RIα gene; when the gene was transfected to liver cells it substantially reduced the level of expression of a number of liver-specific genes [27,28]. Thus the intracellular concentration of RIα subunit can play a crucial role in controlling the expression of sets of tissue-specific genes, even in the absence of activators of the PKA pathway.

Differential expression of RI might of course be expected to affect cellular responses to hormones and other extracellular signaling molecules. The pancreatic beta cell is responsible for regulating blood glucose levels in response to metabolic requirements. Defects in this ability can lead to diabetes [2]. The precise mechanism whereby regulation is achieved is complex and remains poorly understood. The possible connection of the PKA pathway in this process has been examined. Although elevated cAMP levels are insufficient to trigger insulin secretion, cAMP can potentiate glucose-mediated insulin release, presumably a reflection of the biological function of hormones such as glucagon and glucagon-like peptide 1 [18].

The differential expression of RI is likely to have general effects on cell-specific gene expression and on the response of the PKA pathway to extracellular stimuli. Future studies will focus on the downstream targets of PKA, including the potential effects of CREB on key endocrine genes, with a view to understanding how key differences in gene expression patterns and signal transduction events are mediated.

*Acknowledgements:* We thank Dr. R. Seger for many valuable suggestions and critical reading of the manuscript, Dr. L. Journot for kindly providing the CRE-luciferase plasmid and Dr. Helene Edlund, Ulf Ahlgren, Eitan Glick, Kineret Sherman and Sara Weiss for help and valuable discussions. This work was supported by grants to S.S. from the Minerva Foundation, Munich, Germany and the Marcus Sieff Research Fund, and grants to M.D.W. from the Israel Academy of Sciences and Humanities and the Leo and Julia Forchheimer Center for Molecular Genetics at the Weizmann Institute. S.S. is the incumbent of the Kleeman Chair in Biochemistry at the Weizmann Institute of Science. M.D.W. holds the Marvin Meyer and Jenny Cyker Chair of Diabetes Research.

## References

[1] Slack, J.M. (1995) Development 121, 1569–1580.
[2] Efrat, S., Tal, M. and Lodish, H.F. (1994) Trends Biochem. Sci. 19, 535–538.
[3] Philippe, J. (1991) Endocr. Rev. 12, 252–271.
[4] Matschinsky, F.M. (1996) Diabetes 45, 223–241.
[5] Macfarlane, W.M., Smith, S.B., James, R.F., Clifton, A.D., Doza, Y.N., Cohen, P. and Docherty, K. (1997) J. Biol. Chem. 272, 20936–20944.
[6] Heimberg, H., De, V.A., Moens, K., Quartier, E., Bouwens, L., Pipeleers, D., Van, S.E., Madsen, O. and Schuit, F. (1996) Proc. Natl. Acad. Sci. USA 93, 7036–7041.
[7] Ohlsson, H., Karlsson, K. and Edlund, T. (1993) EMBO J. 12, 4251–4259.
[8] Sosa, P.B., Chowdhury, K., Torres, M., Oliver, G. and Gruss, P. (1997) Nature 386, 399–402.
[9] St. Onge, L., Sosa-Pieda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997) Nature 387, 406–409.
[10] Hubank, M. and Schatz, D.G. (1994) Nucleic Acids Res. 22, 5640–5648.
[11] Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. and Baekkeskov, S. (1988) Proc. Natl. Acad. Sci. USA 85, 9037–9041.
[12] Efrat, S., Teitelman, G., Anwar, M., Ruggiero, D. and Hanahan, D. (1988) Neuron 1, 605–613.
[13] Niwa, H., Harrison, L.C., DeAizpurua, H.J. and Cram, D.S. (1997) Endocrinology 138, 1419–1426.
[14] Francis, S.H. and Corbin, J.D. (1994) Annu. Rev. Physiol. 56, 237–272.
[15] Montminy, M. (1997) Annu. Rev. Biochem. 66, 807–822.
[16] McKnight, G.S. (1991) Curr. Opin. Cell Biol. 3, 213–217.
[17] Amieux, P.S., Cummings, D.E., Motamed, K., Brandon, E.P., Wailes, L.A., Le, K., Idzerda, R.L. and McKnight, G.S. (1997) J. Biol. Chem. 272, 3993–3998.
[18] Holz, G.G. and Habener, J.F. (1992) Trends Biochem. Sci. 17, 388–393.
[19] Newgard, C.B. and McGarry, J.D. (1995) Annu. Rev. Biochem. 64, 689–719.
[20] Powers, A.C., Efrat, S., Mojsov, S., Spector, D., Habener, J.F. and Hanahan, D. (1990) Diabetes 39, 406–414.
[21] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1988) Current Protocols in Molecular Biology, Greene/Wiley, Interscience, New York.
[22] Chestukhin, A., Litovchick, L., Batkin, M. and Shaltiel, S. (1996) FEBS Lett. 382, 265–270.
[23] Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P.H. and Journot, L. (1993) Nature 365, 170–175.
[24] Gazdar, A.F., Chick, W.L., Herbert, K.O., King, D.L., Gordon, C.W. and Lauris, V. (1980) Proc. Natl. Acad. Sci. USA 77, 3519–3523.
[25] Neophytou, P.I., Muir, E.M. and Hutton, J.C. (1996) Diabetes 45, 127–133.
[26] Boshart, M., Nitsch, D. and Schutz, G. (1993) Trends Genet. 9, 240–245.
[27] Boshart, M., Weih, F., Nichols, M. and Schutz, G. (1991) Cell 66, 849–859.
[28] Jones, K.W., Shapero, M.H., Chevrette, M. and Fournier, R.E. (1991) Cell 66, 861–872.