ACTH secretion by mouse corticotroph AtT20 cells is negatively modulated by the intracellular level of 7B2

François Bergeron[1], Francine Sirois, Majambu Mbikay*

Diseases of Aging Program, Ottawa Health Research Institute, University of Ottawa, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4K9

Received 29 November 2001; revised 8 January 2002; accepted 8 January 2002

First published online 18 January 2002

Edited by Jacques Hanoune

Abstract 7B2 is a pan-neuroendocrine protein known to facilitate the trafficking and activation of the prohormone proprotein convertase-2 (PC2). 7B2-null mice not only lack PC2 activity, but they also develop an adrenocorticotropic hormone (ACTH) hypersecretion syndrome, suggesting that 7B2 may regulate hormone secretion. To verify this possibility, we introduced into mouse corticotroph AtT20 cells a retroviral vector carrying either a sense or an antisense 7B2 transgene to induce higher and lower 7B2 expression, respectively. Relative to control AtT20 cells, 7B2-overexpressing cells released less ACTH following KCl-induced membrane depolarization, whereas cells expressing lower levels of 7B2 released relatively more, suggesting that 7B2-related peptides modulate regulated secretion in neuroendocrine cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Neuroendocrine protein 7B2; Regulated secretion; Adrenocorticotropic hormone; AtT20 cell

1. Introduction

7B2 is a 21-kDa acidic protein found in the secretory granules of nearly all neuroendocrine cells [1,2]. The structure of its mRNA has been elucidated in many mammalian species by cDNA cloning and sequencing [3–7]. 7B2 is the N-terminal fragment of a 25–29-kDa precursor protein and is generated by endoproteolytic removal of a small carboxyl-terminal peptide [8,9]. This processing is mediated by furin, a member of the proprotein convertase (PC) family of endoproteinases [9].

In neuroendocrine cells, pro7B2 binds the PC2 [10,11] and facilitates its exit from the endoplasmic reticulum, its transport through the Golgi cisternae and its activation in the *trans* Golgi network [12,13]. A central proline-rich domain is responsible for this property [13–15]. The carboxyl-terminal peptide released by furin processing has been shown to be a potent and specific inhibitor of PC2 in vitro [16,17]. Current knowledge on the structure and functions of 7B2 has been recently reviewed [18].

Inactivation of the 7B2 gene in mouse is lethal [19]. Most of the 7B2-null mice die by 3 weeks of age. The brain of these mutant animals contains the 80-kDa proPC2 zymogen but not the 75-kDa active PC2 form nor any PC2 activity. Their pancreatic islets release mostly unprocessed prohormones. Their death is apparently caused by a Cushing-like syndrome characterized by high levels of circulating adrenocorticotropic hormone (ACTH) and corticosterone as well as scapular adipose tissue depots [19]. This syndrome was not observed in PC2-null mice, suggesting that, besides acting as a PC2 chaperone, 7B2 may also influence hormone secretion.

In this study, we have used retroviral vectors to induce high- and low-level production of 7B2 in ACTH-producing AtT20 cells. The results show that the amounts of ACTH released following exocytotic stimulation increases when the cellular content in 7B2 is low and vice-versa, suggesting that 7B2 or its related peptides may play a modulating role in hormone secretion.

2. Materials and methods

2.1. Cell culture

The mouse corticotroph AtT20 cell line was obtained from American Tissue Type Collection. The cells were cultured in Dubelcco's modified Eagle's medium containing 10% fetal calf serum and 4 mM L-glutamine, in a 5% CO_2-humidified atmosphere at 37°C. For exocytotic stimulation, fresh culture medium containing (or not, for control cells) 56 mM KCl and 5.2 mM $CaCl_2$ was substituted and incubation was resumed for 1 h. Metabolic pulse-labeling and immunoprecipitation experiments were conducted as described by Benjannet et al. [11]. All experiments were conducted with triplicate cultures of about 0.5×10^6 cells each. Significant difference between experimental samples was determined by the Student's *t*-test.

2.2. Production of AtT20 cells transduced with a sense or an antisense 7B2 transgene

Recombinant 7B2 retroviruses were prepared as described by Mbikay et al. [20]. Retroviral vectors were constructed by substituting the stuffer sequence in the MNC plasmid with a 1.2-kb full-length mouse 7B2 cDNA. The cDNA was inserted in the sense orientation between *Xma*I and *Not*I or in the antisense orientation between *Hin*dIII and *Not*I, downstream of the human cytomegalovirus immediate early promoter-enhancer (Fig. 1). The MNC plasmid and its 7B2 derivatives were transfected by the calcium precipitation method into DAMP helper cells which contain an encapsidation-deficient Moloney leukemia retrovirus. Transfectants were selected for expression of the neomycin phosphotransferase gene (*neo*^{R}) that confers resistance to the cytotoxic drug G418. Retrovirion-containing culture media were collected. They were used to infect AtT20 cells. Populations of G418-resistant cells that have stably integrated the proviruses were analyzed for 7B2 production and ACTH secretion. The cell populations carry-

*Corresponding author. Fax: (1)-613-761 4355.
E-mail address: mmbikay@ohri.ca (M. Mbikay).

[1] Present address: Laboratoire d'organogénèse expérimentale, Hôpital du Saint-Sacrement, Sainte-Foy, QC, Canada G1S 4L8

Abbreviations: ACTH, adrenocorticotropic hormone; asRNA, antisense RNA; PC, proprotein convertase; RIA, radioimmunoassay

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02277-9

ing the wild-type, the 7B2 sense and the 7B2 antisense provirus are hereafter referred to as RV:WT, RV:7B2S and RV:7B2AS, respectively.

2.3. Real-time quantitative RT-PCR

Total RNA was extracted from cells by the guanidine isothiocyanate extraction method [21]. An aliquot was reverse-transcribed into cDNA using pdN6 random primers and the Superscript II RNase H$^-$ Reverse Transcriptase (Gibco Life Technologies) as previously described [22]. Triplicate aliquots of the cDNA were used in real-time PCRs for 7B2 mRNA (forward primer (F): 5′-GCT GGT CTC TGC TAT GCT ATC T-3′; reverse primer (R): 5′-TTC TAG ACA TCC ATC ATC AGC-3′), 7B2 antisense RNA (asRNA) (F: 5′-GAC CCA AGC TTC TAG AGA TC-3′; R: 5′-GTT TTG GGT TAT GCT GTT TG-3′), or the mRNA for the L30 ribosomal protein as internal standard (F: 5′-AAG TGG GAA GTA CGT GCT GG-3′; R: 5′-CAC CAG TCT GTT CTG GCA TG-3′). The 20-μl reaction mixture contained 1× LightCycler-DNA Master SYBR Green I buffer (Roche; as source of $MgCl_2$, dNTP, SYBR-Green dye and Taq polymerase) supplemented with 2 μl of TaqStart antibody (Clontech) and primers to a final concentration of 0.5 μM each. Fluorometric real-time PCR was conducted using the Roche LightCycler. After a 2-min incubation at 95°C, the reaction mixtures were subjected to 40–45 amplification cycles, each involving a 5-s denaturation at 94°C, a 10-s annealing, a 25-s polymerization at 72°C and a 2-s data acquisition. For 7B2 mRNA, 7B2 asRNA and L30 mRNA, the RT-PCR annealing/acquisition temperatures in °C were 56/80, 58/72 and 49/75, respectively. Standards consisted of varying amounts of 7B2 or L30 pre-quantified cDNA amplicons. Amplicon concentrations were derived at the maximum of the log-linear amplification using the second derivative method [23]. 7B2 RNA levels were normalized for L30 RNA content. For quality control of the cDNA products of each RNA sample, 5 μl was taken from each of the three types of RT-PCR performed on it and combined. The SYBR-Green-stained amplicons were separated by 2.2% agarose gel electrophoresis and visualized by fluorescence.

2.4. Radioimmunoassays (RIAs)

The assays were conducted on culture media and cell extracts. Cells were lysed with a buffer made of 50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholate and 0.1% SDS. The protein content was determined by the Bradford dye method [24]. Antibodies against the $7B2_{23-39}$ peptide and against ACTH as well as the RIAs for the related proteins and peptides have been previously described [1,25]. Results are expressed as picograms and nanograms of immunoreactive (ir) peptide per microgram of intracellular proteins. Values for secreted materials were first corrected for the total volume of culture medium.

3. Results and discussion

Two days after seeding, RV:7B2S cells were morphologically more rounded than RV:WT or RV:7B2AS cells, suggesting that they may have accumulated more secretory materials (Fig. 2a). RV:7B2AS cells grew at a relatively slower rate than the other two cell types. The relative levels of 7B2 mRNA in the three types of cells were assessed by RT-PCR. Compared to RV:WT cells, RV:7B2S cells contained about seven-fold more and RV:7B2AS cells about three-fold less of this mRNA. An RT-PCR specific for 7B2 asRNA expectedly produced an amplicon only with RV:7B2AS cells (Fig. 2b, agarose gel). The presence of this asRNA effectively reduced the steady-state level of 7B2 mRNA, probably by altering either its transcription or its stability.

Metabolic pulse-labeling and immunoprecipitation analysis showed that, relative to RV:WT cells, RV:7B2S cells biosynthesized nearly five-fold more 7B2 and RV:7B2AS cells about two-fold less (Fig. 2c). Since the content of 7B2 mRNA in the latter cells was decreased, the lower level of 7B2 biosynthesis probably reflected this decrease rather than inhibition of 7B2 mRNA translation. Although significant, the decrease was not dramatic, probably because of the limited amounts of asRNA transcripts present in the cells. In view of the growth-reducing effect of the asRNA, it is very likely that, with passages, cells expressing high levels of this RNA would have been selected against in favor of low-expressing ones.

To determine whether changes in 7B2 expression levels had any effect on secretion, we examined regulated release of ACTH-related proteins endogenously produced by these cells. The cells were incubated for 1 h in normal culture medium (basal conditions) or in culture medium supplemented with KCl and $CaCl_2$ to the final concentrations of 56 mM and 5.2 mM, respectively (exocytotic conditions). Materials released under such a short stimulation will most probably consist of pre-existing secretory granule contents rather than products of de novo biosynthesis. Media were collected and titrated by RIA for ir-ACTH. Under basal conditions, the amount in nanograms of secreted ir-ACTH per microgram of intracellular proteins was not significantly different among RV:WT (1.60 ± 0.39), RV:7B2S (1.23 ± 0.12) and RV:7B2AS (1.91 ± 0.58) cells. When the cells were subjected to the exocytotic stimulus, ACTH secretion increased in all three cell types, but not to the same extent. Relative to RV:WT cells (9.2-fold, to 14.65 ± 1.19 ng/μg proteins), the increase was significantly less ($P < 0.05$, 7.8-fold, to 9.62 ± 0.20 ng/μg proteins) in RV:7B2S cells and significantly more ($P < 0.05$, 11.2-fold, to 21.35 ± 2.11 ng/μg proteins) in RV:7B2AS cells (Fig. 3). The intracellular content of pro-opiomelanocortin-related proteins was not significantly different among the three cell types (not shown). The negative correlation between the intracellular levels of 7B2- and ACTH-regulated secretion was not due to any competition for packaging into secretory gran-

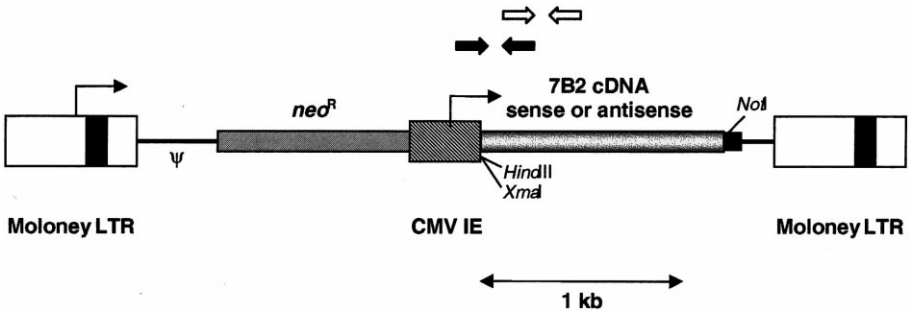

Fig. 1. Diagram of the retroviral expression vectors. The relative positions of the primers used for 7B2 RNA RT-PCR quantification are shown as filled arrows for asRNA and open arrows for the mRNA. Note that the forward primer for the asRNA-specific RT-PCR was derived from the cytomegalovirus immediate early 5′-untranslated region and the reverse primer corresponds to the sense sequence.

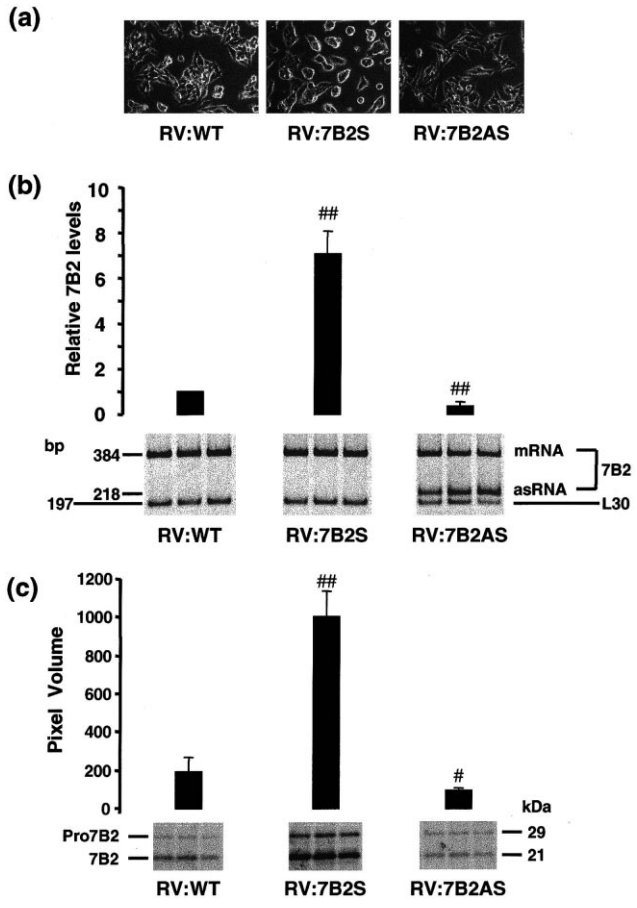

Fig. 2. Expression of 7B2 in the three experimental populations of AtT20 cells. a: Cell morphology. The cells were photographed 48 h after plating and incubation. b: RT-PCR titration of 7B2 mRNA and asRNA. 7B2 mRNA values were normalized relative to those of L30. Histographic representation of 7B2 levels relative to RV:WT cells given the arbitrary values of 1. A qualitative agarose gel electropherogram of the DNA end products is shown below the histogram. c: Biosynthesis of 7B2. The cells were incubated for 1 h in medium containing [^{35}S]methionine and extracted. 7B2-specific immunoprecipitated cells were analyzed by SDS–PAGE and phosphorimaging. The pixel volumes of pro7B2 and 7B2 were quantified and summed up. They are shown in the histogram. Significance relative to RV:WT: $\#P<0.05$; $\#\#P<0.005$.

ules. If that were the case, basal secretion of this hormone should have increased in RV:7B2S and have diminished in RV:7B2AS cells. The very opposite trend was observed when ir-ACTH contents in 24-h spent media were measured. For RV:WT, RV7B2S and RV:7B2AS, these contents were 36.60 ± 3.62, 29.32 ± 1.44 and 48.06 ± 4.07 ng/μg proteins, respectively. These results suggested that 7B2 can negatively modulate ACTH secretion. Absence of this modulatory effect may explain the hypersecretion of ACTH observed in 7B2-null mice [19].

We also compared 7B2 secretion under similar conditions. The results are shown in Fig. 4a. Under basal conditions, the amount of ir-7B2 in 1-h spent media was comparable between RV:WT (29.7 ± 4.9 pg/μg proteins) and RV:7B2AS cells (28.9 ± 2.6 pg/μg proteins), but was nearly 10-fold more for RV:7B2S cells (309 ± 25.5 pg/μg proteins, $P<0.005$). This 10-fold difference between RV:7B2S and RV:WT cells was in discordance with the results of 7B2 biosynthesis which showed intracellular 7B2 in RV:7B2S to be five-fold higher (see Fig.

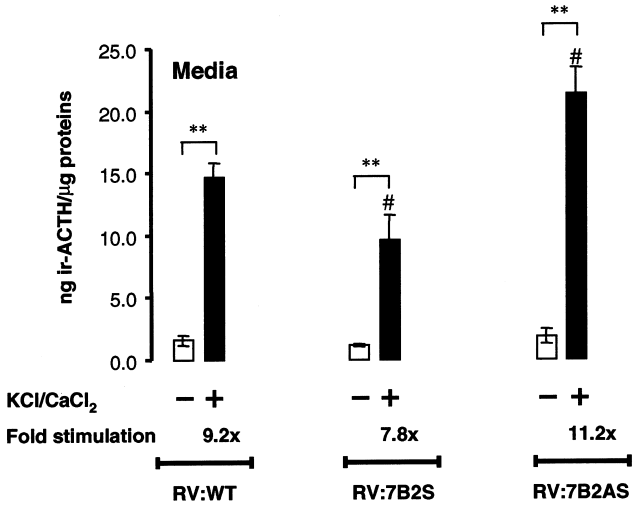

Fig. 3. RIA titration of secreted ir-ACTH before and after KCl/$CaCl_2$-induced membrane depolarization. The level of stimulated secretion is indicated below the bars. White bars, basal levels; black bars, stimulated levels. Significance of difference relative to RV:WT: $\#P<0.05$; or between stimulated and basal levels: $**P<0.005$.

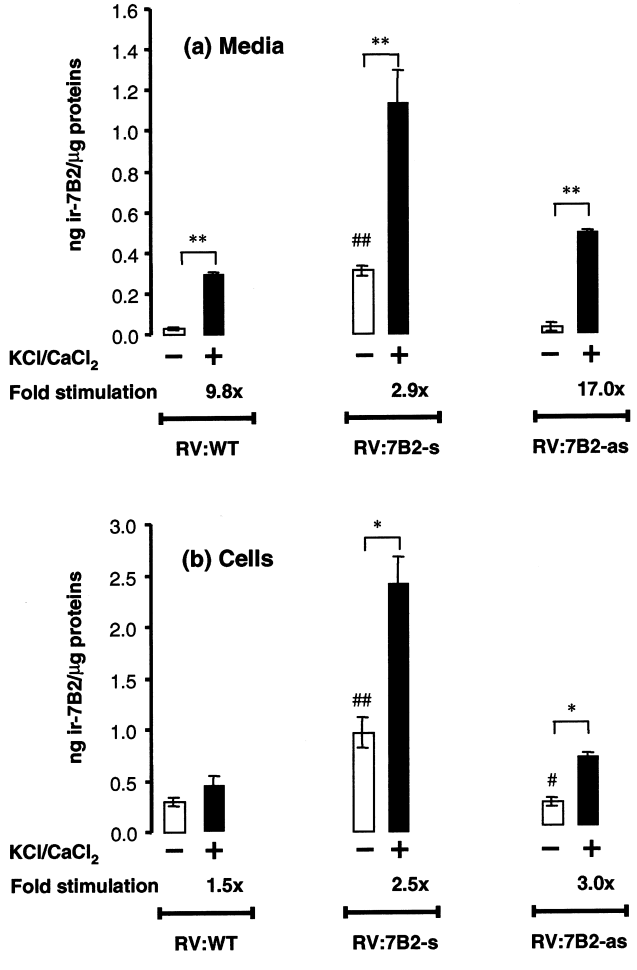

Fig. 4. RIA titration of secreted (a) and intracellular (b) ir-ACTH before and after KCl/$CaCl_2$-induced membrane depolarization. The level of stimulated secretion is indicated below the bars. White bars, basal levels; black bars, stimulated levels. Significance relative to RV:WT: $\#P<0.05$; $\#\#P<0.005$. Significance of difference relative to RV:WT: $\#P<0.05$; $\#\#P<0.005$; or between stimulated and basal levels: $*P<0.05$; $**P<0.005$.

2c). It is yet unclear whether the elevated basal secretion within 1 h after substituting fresh medium is due to some intracellular effect of 7B2 overexpression or to transient relief from an autocrine secretion-countering effect of secreted 7B2 peptides. Exocytotic stimulation increased 7B2 secretion in a pattern similar to that of ACTH. Relative to RV:WT cells which secreted 9.8-fold more ir-7B2 over the basal level (294.1 ± 13.0 versus 29.7 ± 4.9 pg/μg proteins), the response was significantly less with RV:7B2S cells (3.9-fold, 896.8 ± 127.6 versus 309.7 ± 25.5 pg/μg proteins) and significantly higher with RV:7B2AS cells (17-fold, 491.6 ± 16.4 versus 28.9 ± 2.6 pg/μg proteins).

Curiously, after exocytotic stimulation, RV:7B2AS cells secreted in absolute amounts 1.8-fold more 7B2 than control cells (491.6 ± 16.4 versus 294.1 ± 13 pg/μg proteins, $P < 0.005$), although they produced two-fold less (see Fig. 2c). This observation could be explained if 7B2 biosynthesis was rapidly up-regulated by exocytotic stimulation. This was found to be the case for all three cell types (Fig. 4b). The intracellular content in 7B2 in pg/μg of proteins was elevated 1.5-fold in RV:WT cells (297.5 ± 41.1 versus 442.6 ± 97.5, $P < 0.1$), 2.5-fold in RV:7B2S cells (963.0 ± 146.2 versus 2403.6 ± 274.0, $P < 0.05$) and 3-fold in RV:7B2AS cells (222.3 ± 43.7 versus 659.1 ± 38.1, $P < 0.05$). The stimulation was confirmed in pulse-labeling and immunoprecipitation experiments (not shown). It was rapid, as it could be observed within 30 min after exposure of cells to exocytotic medium, suggesting that it occurred at the level of mRNA translation rather than of gene transcription.

To summarize, data presented in this report indicate that the release of ACTH following exocytotic stimulation is higher in AtT20 cells expressing low levels of 7B2 and lower in those expressing high levels of it. We hypothesize that 7B2 or its related peptides act as exocytosis modulators in neuroendocrine cells. At low levels, they accentuate secretion; at high levels, they attenuate it. Their increased biosynthesis under exocytotic conditions may serve to restrain secretion, allowing a faster reconstitution of secretory granule contents. The mechanism of this secretion modulation remains to be determined.

Acknowledgements: The authors thank Dr. Gilles Croissandeau and Dr. Gunther Schmidt for their critical reading of this report. This work was supported by a grant from the Canadian Diabetes Association and the Medical Research Council of Canada.

References

[1] Iguchi, H., Chan, J.S., Seidah, N.G. and Chrétien, M. (1984) Neuroendocrinology 39, 453–458.
[2] Marcinkiewicz, M., Touraine, P. and Chrétien, M. (1994) Neurosci. Lett. 177, 91–94.
[3] Martens, G.J., Bussemakers, M.J., Ayoubi, T.A. and Jenks, B.G. (1989) Eur. J. Biochem. 181, 75–79.
[4] Mbikay, M., Grant, S.G., Sirois, F., Tadros, H., Skowronski, J., Lazure, C., Seidah, N.G., Hanahan, D. and Chrétien, M. (1989) Int. J. Pept. Protein Res. 33, 39–45.
[5] Waldbieser, G.C., Aimi, J. and Dixon, J.E. (1991) Endocrinology 128, 3228–3236.
[6] Spijker, S., Smit, A.B., Martens, G.J. and Geraerts, W.P. (1997) J. Biol. Chem. 272, 4116–4120.
[7] Lindberg, I., Tu, B., Muller, L. and Dickerson, I.M. (1998) DNA Cell Biol. 17, 727–734.
[8] Ayoubi, T.A., van Duijnhoven, H.L., van de Ven, W.J., Jenks, B.G., Roubos, E.W. and Martens, G.J. (1990) J. Biol. Chem. 265, 15644–15647.
[9] Paquet, L., Bergeron, F., Boudreault, A., Seidah, N.G., Chrétien, M., Mbikay, M. and Lazure, C. (1994) J. Biol. Chem. 269, 19279–19285.
[10] Braks, J.A. and Martens, G.J. (1994) Cell 78, 263–273.
[11] Benjannet, S., Savaria, D., Chrétien, M. and Seidah, N.G. (1995) J. Neurochem. 64, 2303–2311.
[12] Barbero, P. and Kitabgi, P. (1999) Biochem. Biophys. Res. Comm. 257, 473–479.
[13] Zhu, X. and Lindberg, I. (1995) J. Cell Biol. 129, 1641–1650.
[14] Muller, L., Zhu, P., Juliano, M.A., Juliano, L. and Lindberg, I. (1999) J. Biol. Chem. 274, 21471–21477.
[15] Van Horssen, A.M. and Martens, G.J. (1998) Mol. Cell. Endocrinol. 137, 7–12.
[16] Martens, G.J., Braks, J.A., Eib, D.W., Zhou, Y. and Lindberg, I. (1994) Proc. Natl. Acad. Sci. USA 91, 5784–5787.
[17] van Horssen, A.M., van den Hurk, W.H., Bailyes, E.M., Hutton, J.C., Martens, G.J. and Lindberg, I. (1995) J. Biol. Chem. 270, 14292–14296.
[18] Mbikay, M., Seidah, N.G. and Chrétien, M. (2001) Biochem. J. 357, 329–342.
[19] Westphal, C.H., Muller, L., Zhou, A., Zhu, X., Bonner-Weir, S., Schambelan, M., Steiner, D.F., Lindberg, I. and Leder, P. (1999) Cell 96, 689–700.
[20] Mbikay, M., Grondin, G., Rondeau, N., Talbot, B.G. and Chrétien, M. (1995) Exp. Cell Res. 220, 79–91.
[21] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[22] Tadros, H., Chrétien, M. and Mbikay, M. (2001) J. Reprod. Immunol. 49, 133–152.
[23] Rasmussen, R. (2001) in: Rapid Cycle Real-Time PCR (Meuer, S., Wittwer, C. and Nakagawara, K., Eds.), pp. 21–34, Springer-Verlag, Berlin.
[24] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[25] Lis, M., Larivière, N., Maurice, G., Julesz, J., Seidah, N. and Chrétien, M. (1982) Life Sci. 30, 1159–1164.