FEBS Letters 557 (2004) 204–208 FEBS 28007

Tyrosine phosphorylation of BIT on photic stimulation in the rat retina

Juri Hamada[a], Nobuaki Okumura[a,*], Miho Inagaki[a], Hiroyuki Taniguchi[a], Yasukazu Nakahata[b], Shin-ichiro Sano[c], Katsuya Nagai[a]

[a] *Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Neuroscience Department, Osaka Bioscience Institute, 6-2-4 Huruedai, Suita, Osaka 565-0874, Japan*
[c] *Mitsubishi Kagaku Institute of Life Science, 11 Minamiooya, Machida, Tokyo 194-8511, Japan*

Received 31 October 2003; revised 5 December 2003; accepted 9 December 2003

First published online 23 December 2003

Edited by Jesus Avila

Abstract BIT is a transmembrane glycoprotein with three immunoglobulin-like domains in its extracellular region and tyrosine phosphorylation sites in its cytosolic region. We have previously shown that BIT was tyrosine phosphorylated in the hypothalamic suprachiasmatic nucleus in response to light exposure during the dark period, and suggested that it was involved in the light entrainment of the circadian clock. To further investigate the function of BIT in the nervous system, we examined the effect of photic stimulation on its tyrosine phosphorylation in the rat retina. It was found that the tyrosine phosphorylation level of BIT in the retina was higher in the light period than in the dark period. In addition, a light stimulation during the dark period resulted in a rapid phosphorylation of BIT and a subsequent association of BIT with SHP-2. The phosphorylation state was quickly reverted when the light was turned off. The light-dependent phosphorylation of BIT was also observed in isolated cultured retinas, and this was blocked by a specific Src-family inhibitor, PP-2. Immunohistochemical study showed that BIT was highly enriched in the inner and outer plexiform layers in the retina, where the immunoreactivity to anti-SHP-2 antibody was also detected. These results suggest that tyrosine phosphorylation of BIT is involved in neuronal transmission in the retina.

Key words: BIT; SHPS-1; SIRP-a; p84; Tyrosine phosphorylation; Retina

1. Introduction

BIT (brain immunoglobulin-like molecule with tyrosine-based activation motifs, also called SHPS-1, SIRP-a and p84) is a transmembrane glycoprotein implicated in intracellular signaling elicited by receptor tyrosine kinases, cell contacts and neuronal transmitters [1–5]. The extracellular region of BIT has three immunoglobulin domains that are thought to interact with extracellular molecules such as CD47/integrin-associated protein [6,7]. Its intracellular domain has two immunoreceptor tyrosine-based inhibitory motifs (ITIM) that are tyrosine phosphorylated on stimulation with various extracellular factors such as epidermal growth factor [4], insulin [3], brain-derived neurotrophic factor [8] and integrins [9]. The phosphorylation of the ITIM motifs of BIT results in association with SHP-2, a protein tyrosine phosphatase that positively regulates the MAP kinase cascade [3,9].

BIT is expressed in several tissues with highest expression levels in the brain, indicating that it has some functional roles in the nervous system [2,3]. We have recently found that BIT is involved in light-induced circadian phase resetting in rats. The circadian clock signal, which is generated by the self-sustained oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is entrained by environmental signals such as the environmental light–dark cycle [10]. We have shown that BIT was tyrosine phosphorylated in the SCN on photic stimulation during the dark period [11]. In addition, administration of anti-BIT monoclonal antibody, which stimulates the tyrosine phosphorylation of BIT [8], into the third cerebral ventricle induced phase shifts of the circadian rhythms of locomotor activity [12]. These results suggest that tyrosine phosphorylation of BIT is involved in the photic entrainment of the master circadian clock in the SCN.

Photic signals evoked in the photoreceptor cells are transmitted either through the direct or indirect neuronal input from retinal ganglion cells to the SCN. These photic signals for the entrainment of the circadian rhythm are transmitted to the SCN at least in part by glutamate as a neurotransmitter [13,14], and in addition, glutamate induced tyrosine phosphorylation of BIT in primary cultures of neuronal cells [12], suggesting a possibility that the light-induced tyrosine phosphorylation of BIT in the SCN might be elicited by glutamate released from the axons of retinal ganglionic cells. This also raised a possibility that activation of other subsets of neurons induces tyrosine phosphorylation of BIT by glutamate or other neurotransmitters in the nervous system.

In response to photic stimulation, the photoreceptor cells of the retina were hyperpolarized by a sequential activation of signaling proteins including rhodopsin, and transducins. The signals were transmitted to the visual cortex via retinal bipolar cells and ganglion cells. In addition, Src-family kinases, which can phosphorylate BIT, have been shown to be activated in the retina on photic stimulation [15]. These facts prompt us to investigate whether BIT is tyrosine phosphorylated in the retina on photic stimulation.

2. Materials and methods

2.1. Antibodies

Rabbit anti-BIT polyclonal antibodies were raised against the recombinant GST-fusion protein of the intracellular domain of BIT

*Corresponding author. Fax: (81)-6-6879 8633.
E-mail address: nokumura@protein.osaka-u.ac.jp (N. Okumura).

0014-5793/03/$30.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01493-5

(399–509). Anti-phosphotyrosine antibody, 4G10, was purchased from Upstate Biotechnology (NY, USA), and anti-SHP-2 antibody was obtained from Santa Cruz (CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was from Cell Signaling (MA, USA), and an ABC antibody detection system was from Vector (CA, USA).

2.2. Photic stimulation of the rat retina in vivo

Five to eight week old male Wistar strain rats were housed in a room illuminated for 12 h (07:00–19:00) daily. In order to stimulate with lights, the rats were exposed to fluorescent light (1000 lx) during the dark period (23:00), and retinas were excised under dim red light. Retinas were then homogenized in a RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM sodium fluoride, 1 mM sodium vanadate, 10 μM sodium molybdate and 10% glycerol. After centrifugation, the supernatant was obtained and its protein concentration was determined by the method of Bradford.

2.3. Photic stimulation of the rat retina in vitro

Rat retinas were excised from Wistar strain rats during the dark period, and kept in DMEM containing 25 mM HEPES at 37°C for 60 min in a dark condition. The retina was then exposed to light (1000 lx) using a fluorescent lamp in the absence and presence of 10 μM PP-2, and homogenized in the RIPA buffer as described above.

2.4. Immunoprecipitation and concanavalin A (ConA) precipitation

Rat retinas were homogenized in the RIPA buffer and centrifuged at $10\,000 \times g$ for 15 min. The supernatants containing 500 μg protein were then subjected to immunoprecipitation using the anti-BIT antibody, followed by Western blotting as described previously [12]. For ConA precipitation, retinal homogenates were incubated with 10 μl of ConA-Sepharose overnight at 4°C.

2.5. Immunohistochemistry

Rat eyeballs were cut out, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) overnight at 4°C and immersed in 30% sucrose for 2–5 days. The eyeballs were then frozen and 10 μm sections were obtained using microtome and stored at 4°C in PBS. All further incubations were performed at room temperature. The sections were treated with a blocking buffer [50 mM Tris–HCl (pH 7.4), 0.9% NaCl, 0.25% gelatin and 0.5% Triton X-100] for 20 min and then incubated with diluted anti-BIT antibody for 1 h. After washing the sections three times with PBS containing 0.03% Triton X-100, the sections were processed with the Vectastain ABC System according to the manufacturer's protocol. The signals were visualized by peroxidase reaction with 3,3-diaminobenzidine as the substrate.

3. Results

3.1. Light-induced tyrosine phosphorylation of BIT in the rat retina

To investigate the effect of light on tyrosine phosphorylation of BIT in the retina, we examined its tyrosine phosphorylation levels during the light period (ZT4) and the dark period (ZT16). ZT refers to zeitgeber time and ZT0 represents the time of light on. We also examined the effect of a photic stimulation during the dark period. At first, we precipitated BIT with ConA-Sepharose because BIT is more effectively precipitated by this method than immunoprecipitation and most of our previous work on the SCN was carried out using this method [11,12]. Next we precipitated BIT by immunoprecipitation with anti-BIT antibody (Fig. 1B). Both of these methods gave basically the same results.

As shown in Fig. 1, the tyrosine phosphorylation level was higher at ZT4 than at ZT16 under the 12 h:12 h light/dark condition. Moreover, when retinas were stimulated with the light during the dark period, tyrosine phosphorylation of BIT was enhanced to a level comparable to that during the light period. Protein levels of BIT in the retina were constant during the light and dark periods. These results suggest that BIT

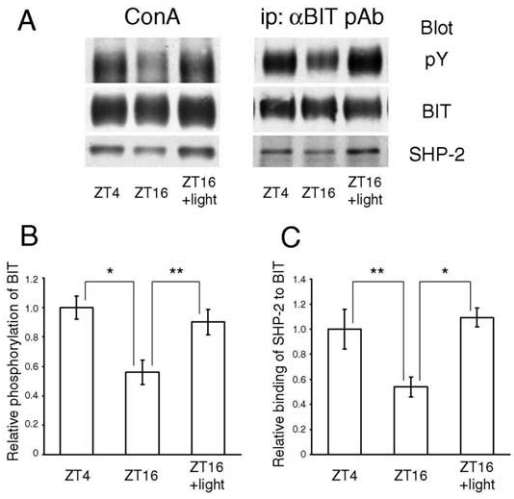

Fig. 1. Light-enhanced tyrosine phosphorylation of BIT in retina. A: Whole rat retinas were obtained at ZT4 and ZT16, as well as ZT16 after 15 min of light exposure in vivo, and subjected to affinity precipitation with ConA-Sepharose or immunoprecipitation with the anti-BIT antibody. The precipitates were then analyzed by immunoblotting with anti-phosphotyrosine (4G10), anti-BIT and anti-SHP-2 antibodies. Similar patterns were observed in four independent experiments. B,C: Quantitative analysis of the immunoblot shown in the right panel of A. Panel B shows tyrosine phosphorylation levels of BIT relative to the amount of BIT in the immunoprecipitates, and panel C shows the amount of SHP-2 relative to the amount of BIT. Data are shown as the means ± S.E.M. of four retinas, and statistical significance is indicated ($*P < 0.05$; $**P < 0.01$).

was tyrosine phosphorylated in the rat retina in response to the photic stimulation.

Tyrosine phosphorylation of BIT induced by growth factors or integrins was shown to result in the association of BIT with SHP-2. To determine whether the light-induced tyrosine phosphorylation of BIT also caused a binding of BIT and SHP-2, ConA-precipitates and immunoprecipitates were further analyzed by Western blotting with anti-SHP-2 antibody. As shown in Fig. 1, association of BIT with SHP-2 was higher in the light period than in the dark period, and, in addition, it was enhanced by the photic stimulation during the dark period. These results suggest that photic stimulation of the retina can induce association of BIT with SHP-2.

Next we examined the time course of light-induced tyrosine phosphorylation of BIT in the retina. Rats were stimulated by a fluorescent light (1000 lx) and tyrosine phosphorylation of BIT in the retina was examined. After the photic stimulation, tyrosine phosphorylation of BIT took place within 5 min (Fig. 2A,B). Its phosphorylation level reached a peak at 30 min and then slightly decreased. We also examined the time course after turning off the light. When the light was turned off, the tyrosine phosphorylation of BIT decreased in 5 min (Fig. 2C,D). In addition, after dephosphorylation in the dark, BIT was phosphorylated again by light exposure (Fig. 2E,F). These results indicate that tyrosine phosphorylation of BIT is probably under a quick regulation by tyrosine kinases and phosphatases.

3.2. Light-induced tyrosine phosphorylation of BIT in vitro and the effect of a Src-family tyrosine kinase inhibitor, PP-2

BIT is a possible substrate for several different protein tyrosine kinases. Among them, we focused on Src-family ki-

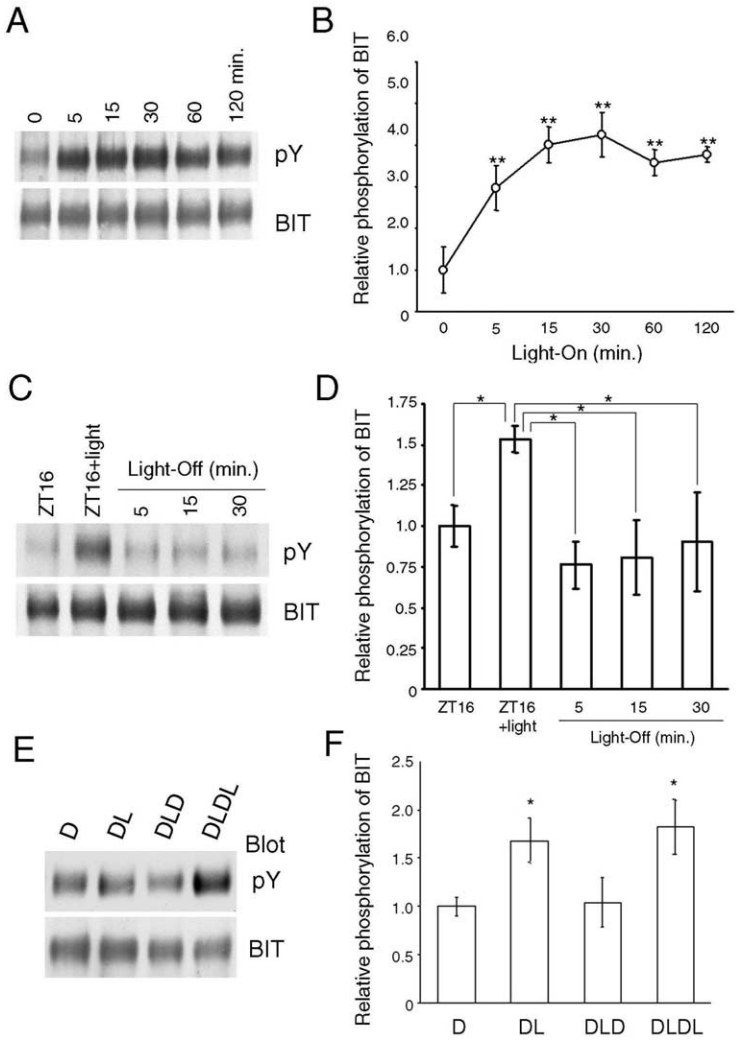

Fig. 2. Time courses of effects of light on and light off on tyrosine phosphorylation of BIT. A,B: Whole rat retinas were obtained at ZT16 before and after light exposure for the indicated times. The samples were then subjected to immunoprecipitation with anti-BIT antibody and immunoblotting with anti-phosphotyrosine antibody. Quantitative analysis of immunoblot in panel A is presented in panel B. C,D: Rat retinas were obtained at ZT16, ZT16 after 15 min of light exposure and subsequently light off. The samples were subjected to the immunoprecipitation with anti-BIT antibody and immunoblotting with anti-phosphotyrosine antibody. Quantitative analysis of the immunoblot shown in panel C is presented in panel D. E,F: Rats kept in the dark (D) were exposed to light for 5 min (DL), then returned to the dark for 5 min (DLD) and exposed to light again for 5 min (DLDL). BIT phosphorylation was examined as above and quantitative analysis is shown in panel F. In panels B, D and F, data are shown as the means ± S.E.M. of four retinas (*$P < 0.05$; **$P < 0.01$).

nases because c-Src was shown to be activated on photic stimulation in the photoreceptor cells in rats [15]. To examine whether Src-family kinases were involved in light-dependent tyrosine phosphorylation of BIT, we tested the effect of PP-2, a specific inhibitor of Src-family tyrosine kinase activities [16], using isolated retinas. As shown in Fig. 3, preincubation of isolated retina with PP-2 reduced the basal phosphorylation level of BIT. After stimulation of the retina with light in vitro, tyrosine phosphorylation of BIT was enhanced about 2.6-fold compared to the basal level. In contrast, it was not enhanced by light in the presence of PP-2. These results suggest that Src-family kinases are the major tyrosine kinases for the basal and light-induced phosphorylation of BIT in the retina.

3.3. Immunohistochemical localization of BIT and SHP-2

To determine the cell types expressing BIT in the retina, we examined immunohistochemical localization of BIT in the retina using the polyclonal anti-BIT antibody (Fig. 4). A dense immunoreactivity was found in the inner and outer plexiform layers, which are synapse-enriched area. On the other hand, immunoreactivity was under the detectable level in the inner nuclear layer and the photoreceptor cell layer.

We also examined the immunohistochemical localization of SHP-2 using anti-SHP-2 antibody and found that it was also localized in the inner and outer plexiform layers. These results are consistent with the idea that BIT is associated with SHP-2 in the retina.

4. Discussion

We have previously shown that BIT is tyrosine phosphorylated in the SCN in response to photic stimulation and suggested its involvement in the light-induced entrainment of the circadian rhythms [11,12]. In the present study, we found that BIT is also tyrosine phosphorylated in the retina in response to photic stimulation, suggesting that the function of BIT in

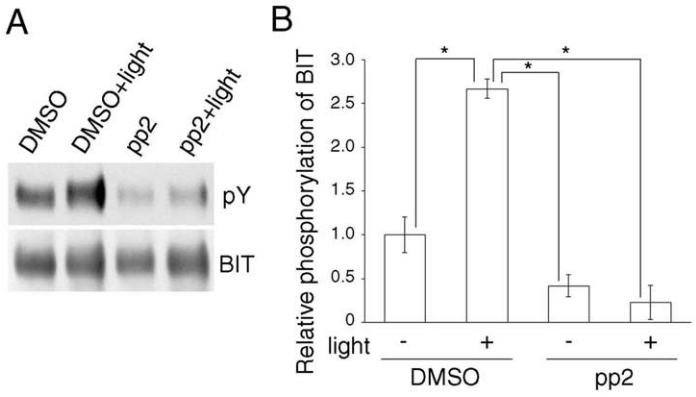

Fig. 3. Effect of a Src-family tyrosine kinase inhibitor, PP-2, on light-induced tyrosine phosphorylation of BIT. A: Rat retinas were obtained at ZT16 under red dim light and transferred into the medium as described in Section 2. After incubating in the absence or presence of 1 μM PP-2 for 15 min at 37°C in the dark condition, retinas were exposed to light for 10 min. The retinas were then subjected to immunoprecipitation with the anti-BIT antibody and immunoblotting with the anti-phosphotyrosine and anti-BIT antibodies. B: Tyrosine phosphorylation levels of BIT relative to its protein levels were analyzed as in Fig. 1B. Data are shown as the means ± S.E.M. of four retinas and the statistical significance is indicated ($*P < 0.05$).

neuronal transmission is not restricted to the SCN neurons. Rather, the present results suggest that BIT is involved in a variety of neuronal activities such as neuronal transmission of light signals in the retina.

Immunohistochemical analysis showed that, in the rat retina, BIT was most abundant in the inner and outer plexiform layers, consistent with the previous report [17]. These regions have a lot of synaptic connections of neuronal cells, and BIT is highly enriched in these synaptic membranes. In addition, BIT is colocalized with synaptic proteins such as PSD95 in primary cultures of neuronal cells prepared from the cerebral cortex (data not shown). It seems thus plausible that BIT has some functional roles at the site of synapses in neuronal transmission of light information.

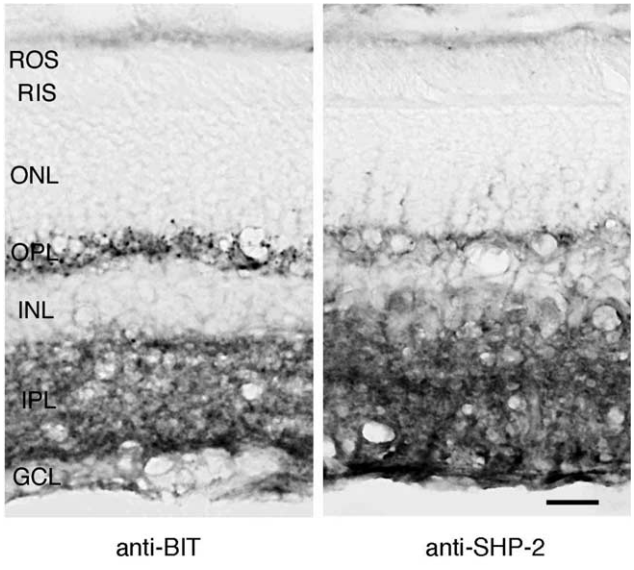

Fig. 4. Localizations of immunoreactivities to anti-BIT and anti-SHP-2 antibodies in the rat retina. Rat retinas were fixed with paraformaldehyde at ZT4 and frozen sections were prepared. These sections were then subjected to immunohistochemical staining with anti-BIT and anti-SHP-2 antibodies. ROS, rod outer segment; RIS, rod inner segment; ONL, outer nuclear layers; OPL, outer plexiform layers; INL, inner nuclear layers; IPL, inner plexiform layers; GCL, ganglion cell layers. Bar: 100 μm.

Using immunoprecipitation with anti-BIT antibody, tyrosine phosphorylation of BIT was shown to result in association with SHP-2 in the retina on photic stimulation. However, the retina is composed of several different types of neurons, and so it was necessary to determine if BIT and SHP-2 are colocalized in vivo. Immunohistochemical analysis showed that SHP-2 was present in the inner and outer plexiform layers and this distribution pattern was quite similar to that of BIT. This suggest that BIT can be actually associated with SHP-2 in the neuronal cells in these areas.

The light-induced tyrosine phosphorylation of BIT was observed within 5 min after photic stimulation, and it was quickly dephosphorylated when light was turned off. This indicates that tyrosine phosphorylation levels of BIT are under quick control by a balance of phosphorylation and dephosphorylation. When eyes were exposed to light, this equilibrium was shifted to phosphorylation, and vice versa. However, it remained to be determined what type of protein phosphatase is responsible for its dephosphorylation.

BIT has been shown to be phosphorylated in response to various growth factors and integrins, suggesting that it can be a substrate for several different protein tyrosine kinases. It has been shown that several Src-family members, including c-Src, Fyn, Lck and c-Yes, were expressed in the retina [15,18–20], and c-Src is activated in response to light in the photoreceptor cells [15]. We have recently found that highly purified recombinant Fyn can directly phosphorylate BIT in vitro (data not shown). In addition, 1D4-induced tyrosine phosphorylation of BIT was blocked by a specific Src-family inhibitor, PP-2 (data not shown). In the present paper, we showed that the tyrosine phosphorylation of BIT by light was blocked by PP-2. Taken together, it was suggested that light-induced tyrosine phosphorylation of BIT might be mediated by activation of Src-family kinases. Among Src-family members, Fyn and Lck have been shown to be present in the outer plexiform layer of the rat retina [19,20], thus these kinases can be candidates for the kinase that phosphorylated BIT on photic stimulation, but other Src-family members may also contribute to it.

Present results raised a possibility that tyrosine phosphorylation of BIT is not only involved in the transmission of light information, but also has a more general function in activated

neurons in the brain. To test this idea, it would be necessary to examine whether BIT is tyrosine phosphorylated in other conditions in which certain types of neurons are activated. In addition, the upstream and downstream signaling of BIT should be studied to clarify the functions of the BIT phosphorylation in the nervous system.

References

[1] Sano, S., Matsuda, M. and Nakagawa, H. (1989) J. Biochem. 105, 457–460.
[2] Sano, S., Ohnishi, H., Omori, A., Hasegawa, J. and Kubota, M. (1997) FEBS Lett. 411, 327–334.
[3] Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T. and Kasuga, M. (1996) Mol. Cell. Biol. 16, 6887–6899.
[4] Kharitonenkov, A., Chen, Z., Sures, I., Wang, H., Schilling, J. and Ullrich, A. (1997) Nature 386, 181–186.
[5] Comu, S., Weng, W., Olinsky, S., Ishwad, P., Mi, Z., Hempel, J., Watkins, S., Lagenaur, C.F. and Narayanan, V. (1997) J. Neurosci. 17, 8702–8710.
[6] Jiang, P., Lagenaur, C.F. and Narayanan, V. (1999) J. Biol. Chem. 274, 559–562.
[7] Seiffert, M., Cant, C., Chen, Z., Rappold, I., Brugger, W., Kanz, L., Brown, E.J., Ullrich, A. and Buhring, H.J. (1999) Blood 94, 3633–3643.
[8] Ohnishi, H., Yamada, M., Kubota, M., Hatanaka, H. and Sano, S. (1999) J. Neurochem. 72, 1402–1408.
[9] Ohnishi, H., Kubota, M., Ohtake, A. and Sano, S. (1996) J. Biol. Chem. 271, 25569–25574.
[10] King, D.F. and Takahashi, J.F. (2000) Annu. Rev. Neurosci. 23, 713–742.
[11] Nakahata, Y., Okumura, N., Shima, T., Okada, M. and Nagai, K. (2000) J. Neurochem. 74, 2436–2444.
[12] Nakahata, Y., Okumura, N., Otani, H., Hamada, J., Numakawa, T., Sano, S. and Nagai, K. (2003) Brain Res. 976, 194–201.
[13] Colwell, C.S., Foster, R.G. and Menaker, M. (1991) Brain Res. 554, 105–110.
[14] Shibata, S., Watanabe, A., Hamada, T. and Ono, M. (1994) Am. J. Physiol. 267, R360–R364.
[15] Ghalayini, A.J., Desai, N., Smith, R., Holbrook, R.M., Elliot, M.H. and Kawakatsu, H. (2002) J. Biol. Chem. 277, 1469–1476.
[16] Hanke, J.F., Gafdner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J. and Connelly, B.A. (1996) J. Biol. Chem. 271, 695–701.
[17] Mi, S.P., Jiang, P., Weng, W.L., Lindbereg, F.P., Narayanan, V. and Lagenaur, C.F.J. (2000) Comp. Neurol. 416, 335–344.
[18] Zhao, Y.H., Baker, H., Walaas, S.I. and Sudol, M. (1991) Oncogene 6, 1725–1733.
[19] Ingraham, C.A., Cooke, M.P., Chuang, Y.N., Perlmutter, R.M. and Maness, P.F. (1992) Oncogene 7, 95–100.
[20] Omri, B., Blancher, C., Neron, B., Marty, M.C., Rutin, J., Molina, T.J., Pesac, B. and Crisanti, P. (1998) Oncogene 16, 2351–2356.