Post-transcriptional inhibition of the interleukin-1 binding protein B15R of Vaccinia virus after coexpression of the related T1 protein

Joana Danescu**, Anne Katrin Werenskiold*

Abteilung Zellchemie, GSF-Forschungszentrum für Umwelt und Gesundheit, Neuherberg, Germany

Received 10 May 1995

Abstract The interleukin-1 binding B15R protein of Vaccinia virus and murine T1 are related extracellular glycoproteins with similarity to the extracellular domain of interleukin-1 receptors. In cells infected with a recombinant Vaccinia virus directing the overexpression of T1, production of the endogenous viral B15R protein is abrogated. T1 synthesis specifically interferes with the production of B15R, but not of other secretory viral proteins. Inhibition of B15R expression occurs at the posttranscriptional level, is exerted in trans and requires the presence of T1 protein in the infected cell. These results suggest a common maturation pathway for the B15R and T1 proteins which might also apply to other members of the interleukin-1 receptor family.

Key words: Interleukin-1 receptor; Immunoglobulin superfamily; Vaccina virus; Post-transcriptional regulation

1. Introduction

The family of interleukin-1 receptor (IL-1R) related proteins comprises four members which are highly related in their extracellular domains [1]. Two of these, the mammalian IL-1 receptors type I and II, are membrane-bound proteins containing unrelated transmembrane and cytoplasmic regions. Two further members are extracellular soluble glycoproteins: B15R, a product of various poxviruses including some strains of Vaccinia virus (VV), exhibits highest homology to the ligand-binding domain of IL-1R type II. The protein has a high affinity to IL-1β, but not IL-1α. Its production in virus-infected cells in vivo interferes with the immune reactions of the infected animal [2].

The second IL-1R related molecule, T1, is most closely related to the ligand-binding domain of IL-1R type I. It was originally identified as a molecule induced by the Ha-*ras* and v-*mos* oncogenes in mouse fibroblasts [3,4] and represents a tumor-associated protein in murine mammary carcinomas [5]. The chromosomal localisation of the T1 gene in close vicinity to the genes encoding IL-1R type I and II both in mouse [6] and man and the conservation of exon/intron boundaries in all three genes [7] suggest a common evolutionary origin of IL-1 receptors and T1. Despite this structural similarity, recombinant T1 protein produced either in insect cells or in mammalian cells does not bind IL-1 cytokines ([8] and herein). Expression of the T1 cDNA from a recombinant Vaccinia virus in mammalian cells directed the synthesis of a secreted, heavily glycosylated protein with a heterogeneous molecular weight of 60 to 70 kDa [9], biochemical properties indistinguishable from those of the endogenous protein in mouse fibroblasts [10].

Experiments performed with T1 protein from cells infected with a recombinant VV revealed a lack of B15R specific IL-1 binding activity. The mechanism leading to the suppression of the synthesis of the virus-encoded B15R protein in the presence of T1 production was analysed here.

2. Materials and methods

2.1 Materials

^{125}I-labeled recombinant human IL-1a and recombinant murine IL-1β were obtained from NEN (Dreieich, Germany, 70–120 μCi/μg).

2.2. Cell culture and VV infection

The construction of the recombinant VV gpt-T1, culturing and infection of RK13 cells have been described earlier [9]. RK13 cells were used in all experiments shown. EL4 cells were grown on RPMI 1640/10% FCS.

2.3. Protein extraction and metabolic labeling

The procedures used for infection and metabolic labeling of VV-infected RK13 cells have been described [9]. Where indicated, proteins were crosslinked by incubation in presence of DSS (freshly solubilized in DMSO) at a final concentration of 1 mg/ml on ice for 40 min. Crude membrane preparations of EL4 cells were obtained as described in [11]. Briefly, cells were washed twice with saline and solubilized in saline containing 1% Triton X-100 and 1 mM PMSF at a concentration of 4×10^8/ml.

2.4. Antisera and immunoprecipitation

The antisera used were the anti-B15R and anti-B18R sera described in [2] and the anti-p9 antiserum specific for the second immunoglobulin-like domain of T1 [9]. Immunoprecipitations were done as described therein. SDS-polyacrylamide gels of radioactive samples were treated with Entensify (NEN, Dreieich, Germany) and dried prior to fluorography.

2.5. IL-1 binding assays

Supernatants of VV infected cells were sterile filtered (0.2 μm pore size) and concentrated tenfold by ultrafiltration (Amicon YM10 membranes) prior to use. The concentrated supernatants of cells infected with either wildtype or gpt-T1 VV were either directly spotted onto ImmobilonP (Millipore Corp., Bedford, MA) membranes (5 μg total protein in 50 μl) or separated in 10% SDS-PAGE without reduction and boiling and transfered to the membrane electrophoretically. Membranes were blocked by incubation in binding buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 2.5% non-fat dry milk) for 15 h at 8°C prior to the addition of [^{125}I]IL-1α or β (5ng/ml) for 2 h at room temperature. After completion of the incubation period membranes were washed extensively in binding buffer and exposed to X-ray film (Fuji) for 3 h to 2 days.

2.6. RNA preparation and analysis

RNA was prepared from the cells as in [5] and the integrity was confirmed by denaturing gel electrophoresis. Reverse PCR analysis was performed as described in detail earlier [5]. Briefly, 1 μg of total RNA

*Corresponding author. *Present address:* GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Molekulare Virologie PO Box 1129, 85758 Oberschleißheim, Germany. Fax: (49) (89) 3187 3329.

**Present address:* GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Säugetiergenetik, Oberschleißheim, Germany.

SSDI 0014-5793(95)00542-0

was reverse transcribed and aliquots of the reaction were used as a template in amplification reactions (30 cycles, annealing temperature 45°C). To exclude false positive signals in reverse PCR due to contamination of the RNA preparation with VV DNA, amplification reactions were run in duplicate from templates prepared with and without addition of reverse transcriptase. At the template concentrations used in the experiments shown here, no amplification products were obtained in the absence of reverse transcriptase. The following primers were used for PCR amplification: B15Rseq: CGCGCCTGAATGTATCGAC; B15Rrev: CTGAGACAGACACGATTGTC (nucleotide positions 186–204 and 482–462, respectively, according to the sequence as published in [12]) B18Rseq: GGTACTCATGATAAGTATGGCATAGAC; B18Rrev: GTGGTCTTGTGACGGTATAACC (nucleotide positions 721–747 and 990–969, respectively according to the sequence as published in [12]).

3. Results

3.1. Lack of IL-1β binding activity in cells infected with a T1 expressing VV

The construction of the T1 expressing recombinant VV (gpt-T1) has been described [9]. Briefly, the coding region of the T1 cDNA was subjected to the control of the strong viral late promoter 11k and inserted into the viral TK-gene by homologous recombination with the VV strain Western Reserve. The Western Reserve strain of VV (hereafter designated wt VV)

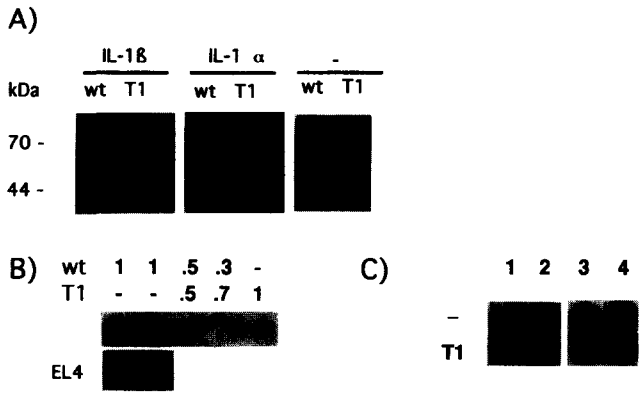

Fig. 1. IL-1 binding activity in the supernatants of RK13 cells infected with wt or gpt-T1 VV. **(A)** Proteins from concentrated supernatants of wt (lanes **wt**) or gpt-T1 (lanes **T1**) VV infected cells harvested 24 h post-infection were separated in non-reducing 10% SDS-PAGE, transfered to nylon membranes, incubated with [^{125}I]IL-1β or [^{125}I]IL-1a as indicated and the washed membranes were exposed to X-ray film. After autoradiography the membrane was hybridised to a T1 specific antiserum to demonstrate the position of T1 on the filter (lanes −). **(B)** RK13 cells were infected with wt (**wt**) VV, gpt-T1 (**T1**) or a mixture of both. Supernatants were harvested 24 h post-infection. The relative amount of each virus was determined by Southern blot analysis of viral DNA isolated from the infected cells and is indicated at the top of the figure. Concentrated, sterile filtered native supernatants (20 μl) were dotted onto nylon membranes along with 10 and 20 μl of crude membrane preparations of EL4 cells (**EL4**) (corresponding to 4×10^6 and 8×10^6 cells) used as positive control. The membrane was reacted with [^{125}I]IL-1β, washed and exposed to X-ray film. **(C)** Concentrated supernatant of wildtype VV infected cells was incubated in vitro for 2 h at 37°C in the presence (**T1**) or absence (−) of an equivalent amount of medium containing recombinant T1 (**lanes 1,2**). Cells infected with wildtype VV were incubated in either fresh serum free medium (−) or in virus-free medium containing exogenous T1 produced by gpt-T1 infected cells (**T1**) for 24 h after infection (**lanes 3,4**). Aliquots corresponding to 5 μl (**lanes 1,3**) and 20 μl (**lanes 2,4**) of concentrated supernatant were dotted on the nylon membrane and reacted with IL-1β as above.

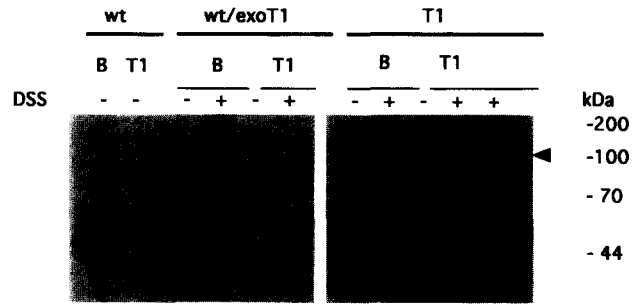

Fig. 2. Absence of the B15R protein in the media of gpt-T1 infected cells. RK13 cells were infected with wildtype VV and incubated in the absence (**wt**) or presence (**wt/exoT1**) of unlabeled exogenous T1 protein or infected with gpt-T1 (**T1**) virus. Metabolic labeling was performed from 15 to 19 h post-infection. Harvested media were either left untreated (lanes −) or crosslinked with DSS (lanes +) prior to precipitation with antisera specific for the T1 (lanes **T1**) or B15R (lanes **B**) proteins. The precipitated proteins were separated in 10% SDS-PAGE and fluorographed. The additional band precipitated by anti-T1 serum from the medium after crosslinking is indicated by the arrowhead.

directs the expression of the viral IL-1β binding B15R protein in infected cells. Consistent with earlier reports [2,13], the analysis of IL-1a and β binding activities in supernatants of cells infected with wt VV by ligand blotting revealed the presence of a protein of approx. 45 kDa, B15R, which exclusively bound IL-1β but not a. In the media of gpt-T1 VV infected cells, however, IL-1 binding activity could not be observed at either the position of the B15R or the T1 proteins. The presence of T1 on the membrane was confirmed by hybridisation with T1 specific antiserum after the ligand blotting procedure (Fig. 1A). These experiments indicated that (i) the T1 protein was incapable of binding IL-1 under these conditions and (ii) the B15R protein either had lost the ability to bind the cytokine or was absent from supernatants of gpt-T1 infected cells.

In a second set of experiments cells were infected either with purified wt or gpt-T1 VV or a mixture of both. To assess the ratio of both VVs in the individual infection, viral DNA was isolated from the cells after the incubation period and analysed by Southern blotting (not shown). Binding assays were performed with native supernatant proteins immobilized on a nylon membrane using a dot blot technique. As shown in Fig. 1B, binding of IL-1β was apparent in the supernatants of wildtype VV infected cells. In the supernatants of cells infected with gpt-T1 virus alone or in combination with wt VV, IL-1 binding activity was not detected.

The loss of B15R activity in the presence of T1 may be due to a direct interaction of the T1 and B15R proteins in the supernatant. If so, the addition of recombinant T1 to supernatants of cells infected with wt VV should elicit the same effect. To test this assumption, isolated concentrated supernatants of wt VV infected cells containing active B15R protein were incubated for 2 h at 37°C in the absence or presence of equal amounts of supernatant from gpt-T1 infected cells prior to the analysis of IL-1β binding activity (Fig. 1C, lanes 1,2). Further, virus-free supernatant from gpt-T1 infected cells was added to cells infected with wt VV throughout the infection period. The IL-1β binding activity of the B15R protein was not affected by incubation in presence of T1 in both experimental set ups (Fig. 1C, lanes 3,4).

3.2. T1 inhibits the secretion of B15R

The presence of B15R protein in the supernatants of VV infected cells and its ability to form complexes with T1 were assessed by immunoprecipitation. Since both B15R and the recombinant T1 are expressed from late viral promoters [9,12], metabolic labeling was performed at various intervals during the late phase of infection. Consistently B15R protein could be precipitated from media of cells infected with wt VV, but was absent from supernatants of gpt-T1 virus infected cells (Fig. 2, lanes B). The presence of exogenously added T1 in the supernatant of wt VV infected cells (Fig. 2, lanes wt/exoT1) did not affect the amount of precipitable B15R protein. Furthermore, covalent crosslinking of proteins in the supernatant prior to immunoprecipitation demonstrated the monomeric nature of B15R in the presence of T1, thus excluding the possibility that the apparent absence of B15R from T1-containing media was due to masking by complex formation. Higher molecular weight complexes were only observed after precipitation of crosslinked supernatants of gpt-T1 virus infected cells with anti-T1 antisera. The additional band of 130–145kDa molecular weight obviously represents a T1 homodimer, since (i) it conforms to the expected size of 120–140 kDa and (ii) no radiolabeled product was coprecipitated with the antiserum when media of wildtype VV infected cells were crosslinked in presence of unlabeled exogenous T1. Taken together these data suggested that the B15R protein is not secreted from cells infected with gpt-T1 virus.

3.3. The inhibition of B15R secretion is specific

During the late phase of VV infections an exhaustion of the cellular secretion machinery ('secretory overload') may occur and inhibit protein maturation and export. Therefore the expression of two further extracellular viral proteins was analysed. The major secreted protein of VV, a 35 kDa protein with homology to complement regulatory factors [14] may easily be identified after gel-electrophoretic separation of radiolabeled proteins from the supernatants of cells infected with VV. Supernatants of cells infected with either wt- or gpt-T1 virus metabolically labeled from 15 to 19 h post-infection contained similar amounts of this highly expressed protein (Fig. 3A). Pulse-chase

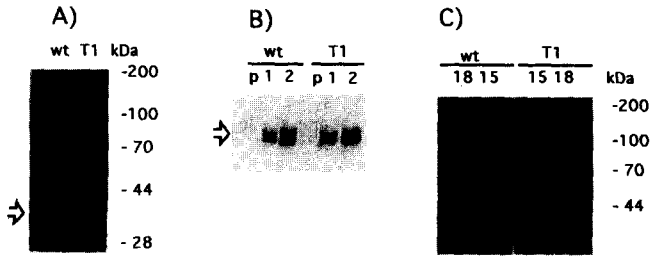

Fig. 3. Secretion of VV-encoded 35kD and B18R proteins from infected cells. **(A)** Supernatants of cells infected with either wt (**wt**) or gpt-T1 VV (**T1**) were metabolically labeled from 15 to 19 h post-infection and resolved in 10% SDS-PAGE. The position of the 35 kDa major secreted protein is indicated by an arrow. **(B)** Pulse-chase analysis for secretion of the 35 kDa protein. Cells were infected as above, labeled from 15 to 16 h post-induction and media were collected and analysed in 10% SDS-PAGE either directly after the labeling period (**p**) or after a chase for 3 h (**1**) or 7 h (**2**). Only the relevant part of the gel is shown, the 35 kDa band is indicated by the arrow. **(C)** Cells were infected and labeled as in **(A)** and the supernatants were precipitated with antisera directed against the B15R (**15**) or B18R (**18**) proteins. A fluorograph of the 10% SDS-PAGE is shown.

experiments were performed to study the kinetics of secretion of the 35 kDa protein in detail. Infected cells were metabolically labeled from 15 to 16 h after infection and the total secreted proteins were analysed either directly after the labeling period or after a chase for 3 or 7 h. As depicted in Fig. 3B, the amount of the 35 kDa component and the kinetics of its secretion were indistinguishable in cells infected with either virus.

The second extracellular protein studied for expression in gpt-T1 virus infected cells, B18R, is another glycoprotein of the immunoglobulin superfamily. It bears a sequence homology to B15R of 20% and is synthesized in relatively low amount [12]. Supernatants of cells infected with wt VV and metabolically labeled from 15 to 19 h post-infection contained similar amounts of immunoprecipitable B18R and B15R molecules. After infection with gpt-T1 virus, B18R – but not B15R – protein was precipitated from the supernatant (Fig. 3C).

3.4. B15R transcription

To evaluate the contribution of transcriptional events to the inhibition of B15R synthesis, levels of B15R transcripts were analysed by reverse PCR. Total RNA was prepared 18h after infection of cells with either wildtype VV or the recombinant gpt-T1 or a 1:1 mixture of both. IL-1β binding was only detected in the supernatant of cells infected with wt virus (not shown). The RNA preparations were reverse transcribed and PCR analysis was performed on serial dilutions of the cDNAs in the presence of primers specifying B15R and B18R coding sequences. At the dilutions selected, the amount of the expected amplification products (B15R: 295 bp, B18R: 271 bp) increased linearly with the amount of template added. No difference in the relative amount of B15R and B18R transcripts was observed in RNA from cells infected with the different VV (Fig. 4).

4. Discussion

In this contribution we describe the selective inhibition of B15R synthesis in presence of the structurally related T1 protein in VV infected cells. Similar amounts of B15R transcripts were present in cells infected with either wildtype VV or the recombinant gpt-T1 virus or a 1:1 mixture of both, confirming that the presence of T1 sequences in the recombinant gpt-T1 virus does not affect the level of the B15R transcript. Production of active B15R protein, however, was only detected in cells infected with wildtype VV. Cells infected either exclusively with the recombinant gpt-T1 virus or a combination of the recombinant and wildtype VV failed to produce B15R protein, indicating that the inhibition of B15R expression is exerted in trans.

The structural similarity of the T1 and B15R glycoproteins may suggest a direct molecular interaction of both molecules resulting in the formation of heterodimers/oligomers lacking affinity to IL-1. Several experiments rule out this notion: (i) An incubation of the mature secreted B15R protein with T1 in cell culture supernatant for prolonged periods of time did not affect IL-1β binding by B15R. (ii) Supplementation of the medium of cells infected with wt VV with T1 protein throughout the incubation period led to production of active B15R protein. (iii) Immunoprecipitation of covalently crosslinked proteins present in the supernatant of the infected cells confirmed the presence of both T1 and B15R in monomeric form. In addition it demonstrated the absence of immunoprecipitable B15R pro-

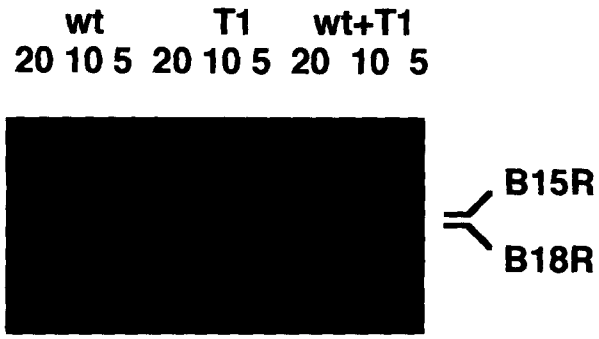

Fig. 4. Presence of B15R and B18R transcripts in VV infected cells. RK13 cells were infected with either wt (**wt**) or gpt-T1 (**T1**) VV or a 1:1 mixture of both (**wt+T1**). Total RNA prepared from the cells 18 h post-infection was reverse transcribed. Serial dilutions of the cDNA preparations were analysed by PCR in the presence of primer pairs specifying B15R (295 bp amplification product) and B18R RNA (271 bp amplification product). The amplification products obtained from cDNA corresponding to 20, 10 or 5 ng of RNA template as indicated were separated in a 2% agarose gel, an ethidium bromide stain is shown.

tein from supernatants of cells infected with gpt-T1 virus. Taken together these data show that the inhibition of B15R expression depends on an intracellular presence/synthesis of T1 protein.

The observed post-transcriptional inhibition of B15R production may be reconciled with two different levels of regulation. Since an accumulation of intracellular B15R protein was not observed in cells infected with gpt-T1 virus (not shown), overexpression of T1 may interfere with translational initiation at B15R mRNA. Although the mechanisms regulating translational initiation remain incompletely understood to date, specific protein–RNA interactions required for translational activation of selective mRNA species have been identified (reviewed in [15]). The high level of T1 mRNA present in the infected cells might compete with B15R mRNA for a (hypothetical) initiation factor essential for translational activation of either transcript. However, a comparison of the 5′ non-translated sequences of both transcripts failed to reveal related motifs.

Alternatively, T1 expression might interfere specifically with the posttranslational maturation of B15R. We found earlier that fully processed, complex glycosylated T1 protein started to accumulate in gpt-T1 VV infected cells 18h post infection [9], whereas the endogenous fully processed T1 protein produced in fibroblasts was quantitatively secreted [10]. This discrepancy suggested that the expression of recombinant T1 at high level depleted the cellular stores of the lethally infected cells for an essential accessory folding proteins/chaperone. The current finding that the production of B15R, but not of B18R and the 35 kDa major secreted protein of VV, is inhibited in the presence of high level T1 synthesis likely indicates a competition of nascent B15R and T1 molecules for a common maturation factor in the infected cell. An important role of molecular chaperones in synthesis, translocation, folding and degradation of secretory proteins has been suggested [16–18] and recent examples provide evidence for a sequential function of different chaperones in the endoplasmic reticulum of mammalian cells [19,20]. Conceivably, this sequential action of chaperones requires an intricate balance in the endoplasmic reticulum for efficient function which is lost in VV infected cells. The utilization of this exhaustible system thus facilitated the first identification of a common maturation pathway for two proteins of the IL-1 receptor family. Further work will have to define this common folding/maturation pathway of B15R and T1 proteins in detail. These studies will also reveal whether the same pathway is utilized in the maturation of the other members of this protein family – the mammalian Interleukin-1 receptors.

Acknowledgements: We wish to thank Dr. A. Alcami for the anti B15R and anti B18R antisera and M. Löwel for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Grant We1375/2-2)

References

[1] McMahan, C.J. Slack, J.I., Mosley, B., Cosman, D., Lupton, S.D., Brunton, L.L., Grubin, C.E., Wignall, J.M., Jenkins, N.A., Copeland, N.G., Huebner, K., Croce, C.M., Cannizzarro, L.A., Benjamin, D., Dower, S.K., Spriggs, M. and Sims, J.E. (1991) EMBO J. 10, 2821–2832.
[2] Alcami, A. and Smith, G.L. (1992) Cell 71, 153–167.
[3] Werenskiold, A.K., Hoffmann S. and Klemenz, R. (1989) Mol. Cell. Biol. 9, 5207–5214.
[4] Klemenz, R., Hoffmann, S. and Werenskiold, A.K. (1989) Proc. Natl. Acad. Sci. USA 86, 5708–5712.
[5] Rößler, U., Andres, A.C., Reichmann, E., Schmahl, W. and Werenskiold, A.K. (1993) Oncogene 8, 609–617.
[6] Yanagisawa, K., Tsukamoto, T., Takagi, T., Tetsuka, T. and Tominaga, S. (1993) FEBS Lett. 318, 83–87.
[7] Sims, J.E., Painter, S.L. and Gow, I.R. (1995) Cytokine in press,
[8] Rößler, U., Thomassen, E., Hültner, L., Baier, S., Danescu, J. and Werenskiold, A.K. (1995) Dev. Biol. 168, 86–97.
[9] Werenskiold, A.K. (1992) Eur. J. Biochem. 204, 1041–1047.
[10] Thomassen, E., Kothny, G., Haas, S., Danescu, J., Hültner, L., Dörmer, P. and Werenskiold, A.K. (1995) Cell Growth Differ. 6, 179–184.
[11] Urdal, D.L., Call, S.M., Jackson, J.L. and Dower, S. K. (1988) J. Biol. Chem. 263, 2870–2877.
[12] Smith, G.L. and Chan, Y.S. (1991) J. Gen. Virol. 72, 511–518.
[13] Spriggs, M.K., Hruby, D.E., Maliszewski, C.R., Pickup, D.J., Sims, J.E., Buller, R.M. L. and VanSlyke, J. (1992) Cell 71, 145–152.
[14] Kotwal, G.J., Isaacs, S.N., McKenzie, R., Frank, M.M., and Moss, B. (1990) Science 250, 827–830.
[15] Kaufman, R.J. (1994) Curr. Opin. Biotech. 5, 550–557.
[16] deSilva, A.M., Balch, W.E. and Helenius, A. (1990) J. Cell Biol. 111, 857–866.
[17] Gething, M.J. and Sambrook, J. (1992) Nature 355, 33–45.
[18] Urade, R., Takenaka, Y. and Kito, M. (1993) J. Biol. Chem. 268, 22004–22009.
[19] Melnick, J., Dul, J.L. and Argon, Y. (1994) Nature 370, 373–375.
[20] Kim, P.S. and Alvan, P. (1995) J. Cell Biol. 128, 29–38.