FEBS Letters 412 (1997) 370–374 FEBS 18955

Isolation of mRNAs induced by a hazardous chemical in white-rot fungus, *Coriolus versicolor*, by differential display

Yosuke Iimura*, Kenji Tatsumi

Department of Hydrospheric Environmental Protection, National Institute for Resources and Environment, AIST, MITI, 16-3 Onogawa, Tsukuba, Ibaraki 305, Japan

Received 30 May 1997

Abstract White-rot fungus *Coriolus versicolor*, a ligninolytic basidiomycete, has been studied because of its ability to degrade hazardous chemicals. In this study, we searched for genes that are induced by a hazardous chemical using the mRNA differential-display technique and *C. versicolor* IFO30340 that has been exposed to pentachlorophenol (PCP). Five cDNA fragments were cloned and the DNA sequences of two fragments were analyzed in further detail. The clones corresponded to novel genes that have not previously been identified in *C. versicolor*. One of the cDNAs exhibited strong sequence homology to the gene for an enolase and the other exhibited homology to a heat shock protein. The expression of the two genes was up-regulated in PCP-treated *C. versicolor*.

Key words: White-rot fungus; Basidiomycete; Hazardous chemical; Differential display; Heat shock protein; Enolase

1. Introduction

White-rot fungi, *Phanerochaete chrysosporium* and *Coriolus versicolor*, are typical lignin-degrading microorganisms. The lignin-degradation system of the fungi consists of non-specific oxidative reactions that are catalyzed by multiple isozymes of phenoloxidase [1–7]. Since white-rot fungi have a system for the degradation of lignin, it has been proposed that these fungi shown be useful for bioremediation [8,9]. Indeed, the ligninolytic systems of these fungi can degrade a wide variety of aromatic pollutants [10–12].

The cloning, and characterization of the genes that encode the various ligninolytic enzymes have been reported [13–17], and the regulation of the genes has been examined in detail [18–21]. Expression of one of the ligninolytic enzymes, manganese peroxidase of *P. chrysosporium*, is regulated at the transcriptional level by nutrients, manganous ions, oxidative stress, and chemical stress [22]. Lignin peroxidase genes and manganese peroxidase gene have been also cloned from *Trametes* (*Coriolus*) *versicolor* [23,24]. The promoter regions of these genes contain putative heat shock elements and metal response elements similarly to those of *P. chrysosporium*. It can therefore be presumed that these peroxidases of *T. versicolor* are regulated by chemical stress, by analogy to the observation in *P. chrysosporium*. Understanding hazardous chemicals-degradation by the white-rot fungi at a molecular level might be useful for future applications of the organisms to bioremediation. However, more information about the degradative mechanism, as well as details of the physiological and enzymatic regulation of the degradation, is needed for full realization of the potential utility of these organisms to bioremediation.

In order to understand the responses of fungi to environmental stress and to identify changes in gene expression, we used the differential-display technique [25] to identify genes that are differentially expressed in chemically stressed *C. versicolor*.

2. Materials and methods

The fungal strain used in this study was *Coriolus versicolor* IFO30340. This fungus was grown at 28°C in nitrogen-limited liquid culture (LN) [26]. Pentachlorophenol (PCP), dissolved in dimethyl formamide (DMF), was used at a final concentration of 100 μM.

Six-day-old, nitrogen-limited liquid cultures were incubated with or without PCP. In each case, the vehicle (DMF) was present at 0.5% (v/v). The cells were harvested after 4 h, and mRNA was extracted with a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). cDNA was synthesized from mRNA with a RTG You-Primed First-Strand Kit (Pharmacia Biotech). The three anchored primers used were 5′-GT_{15}A-3′, 5′-GT_{15}C-3′, and 5′-GT_{15}G-3′, (abbreviated as GT_{15}V). The samples were diluted 5-fold.

Subsequent amplification by PCR of cDNAs was performed with the appropriate GT_{15}V in combination with different 18-base arbitrary primers obtained from Stratagene. The conditions for PCR, that were based on Ito et al. [27] with some modification, were as follows: 1.0 U of GeneTaq polymerase (Nippon Gene), 0.22 μg of TaqStart Antibody (Clontech), 1× GeneTaq buffer (Nippon Gene), 0.5 μM arbitrary primer, 0.5 μM anchored primer, 200 μM dNTPs, and 1 μL of the solution of diluted cDNA in a final volume of 20 μL. The temperature profile was as follows: one set of incubations at 94°C for 5 min, at 36°C for 5 min, and at 72°C for 5 min, which was followed by 30 high-stringency cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. All amplifications by PCR were performed in a DNA Thermal Cycler 2400 (Perkin-Elmer). The amplified products were then separated electrophoretically on an ExelGel (Pharmacia Biotech). For detection of bands of cDNAs, the gel was stained with a DNA Silver-Staining Kit (Pharmacia Biotech).

Differentially amplified fragments were excised from the gel and redissolved in sterile water. Reamplification by PCR was performed with the appropriate pair of primers. Fragments were cloned into the TA site of pCR2.1 (Invitrogen) and were sequenced with an automated DNA Sequencer (ALFredII; Pharmacia Biotech) using Thermo Sequenase (Amersham). DNA sequences were analyzed by the FASTA or BLAST search programs. A full-length cDNA for the DD123 gene and a partial cDNA for the DD13 gene were obtained by the rapid amplification of cDNA ends (RACE) method [28] with a Marathon cDNA Amplification Kit (Clontech). The sequence of the FDD123 gene of *Coriolus versicolor* reported in this paper has been assigned DDBJ accession number AB003518.

Total RNA was also extracted from *C. versicolor* that has been exposed to PCP with an RNeasy Plant Total RNA Kit (Qiagen) for analysis by reverse transcription-PCR (RT-PCR). cDNA was synthesized from total RNA with an RTG T-Primed First-Strand Kit (Pharmacia Biotech). The resultant cDNA was subjected to 20 cycles of amplification by PCR. The amplification mixture contained (in a final volume of 20 μl) 1.0 U of LA Taq polymerase (Takara Biomedicals), 0.22 μg TaqStart Antibody (Clontech), 1× LA Taq buffer ($MgCl_2$ plus), 200 μM dNTPs, and 1.0 μM each primer. Oligonucleotides used

*Corresponding author. Fax: (81) (298) 58-8308.
E-mail: iimura@nire.go.jp

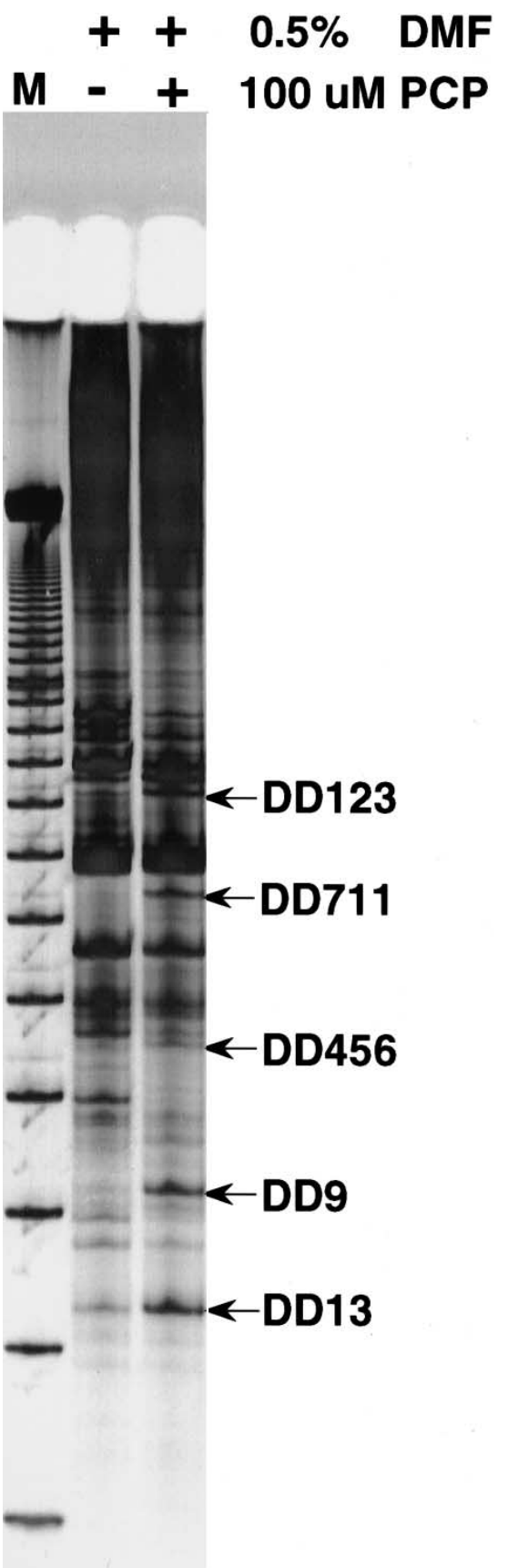
PII S0014-5793(97)00807-7

as primers for PCR were 5′-GTTACAATCTATGAGCACAG-CGAC-3′ and 5′-ACCAGATCGGTACCATCTCGGAG-3′ for DD135, and 5′-GACCTCAACCCTCCCAACGCTA-3′ and 5′-CA-GCCAGGATGTAGCCGCGAC-3′ for FDD123, respectively. The temperature profile for PCR was as follows: 20 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The amplified products were separated on a 2% agarose gel. The gel was stained with SYBR Green I (Molecular Probes) and visualized with FluorImager 595 (Molecular Dynamics). The intensities of bands were determined with Image-Quant software (Molecular Dynamics).

3. Results and discussion

C. versicolor was treated with pentachlorophenol (PCP) and cDNAs were isolated by the differential display method. Differential display patterns from control and PCP-stressed *C. versicolor* indicated that many bands were common to both samples and the remainder were specific either to the control or to the PCP-treated sample (Fig. 1). The reproducibility of the fingerprint pattern was satisfaction. Three specific cDNA fragments (DD711, DD456, and DD9) derived from transcribed mRNA were identified, and the intensities of signals due to two fragments (DD123, and DD13) were increased by exposure of the fungus to PCP (indicated by arrows). These fragments were excised from the gel and reamplified with 5′-AATCTAGAGCTCCTCCTC-3′ as the 5′ primer and $GT_{15}G$ as the 3′ primer. The products were inserted into the TA vector. Clones bearing inserts comigrating precisely with the band of interest were subjected to DNA sequencing. Out of five cDNA fragments sequenced, three cDNAs exhibited no significant homology to any sequence in the gene databases (data not shown). However, the homology search revealed that the clone designated DD13 exhibited significant homology to a cDNA for enolase (ENO) while DD123 exhibited homology to a cDNA for heat shock protein 30 of *Saccharomyces cerevisiae* (HSP30). The clones represented genes that had not previously been identified in *C. versicolor*. We used the RACE method to generate full-length cDNAs for DD123 and DD13.

The nucleotide sequence of the cloned full-length cDNA (FDD123) derived from DD123, as well as the deduced amino acid sequence, is shown in Fig. 2. The sequence is 1207 nucleotides long and contains an open reading frame that starts with an ATG codon at position 63 and extends as far as the TAA triplet at nucleotide 912. The open reading frame encodes a protein of 283 amino acids with a predicted molecular weight of 30,991 Da. This gene has a high G+C content (60.1%) and the frequency of occurrence of G or C at the third position of each codon is 65%. The amino acid sequence is that of a very hydrophobic protein (Fig. 2), resembling the



→

Fig. 1. Differential display of mRNAs isolated from *C. versicolor* cells that had been incubated with or without pentachlorophenol (PCP). Cells in six-day-old, nitrogen-limited cultures were incubated with or without PCP. Cells were harvested after 4 h and mRNA was extracted. cDNA was synthesized from the mRNA. The anchored primer used was 5′-GTTTTTTTTTTTTTTTG-3′ ($GT_{15}G$). Subsequent amplification of cDNAs by PCR was performed with the $GT_{15}G$ in combination with an 18-base arbitrary primer (5′-AATCTAGAGCTCCTCCTC-3′). Lane M was loaded with the 100 bp ladder of DNA (Pharmacia Biotech) for molecular weight marker.

Fig. 2. Nucleotide and deduced amino acid sequences of FDD123. A full-length cDNA for the DD123 gene was obtained by the RACE method. Solid lines indicate the primers for analysis by RT-PCR. Boxes indicate potential transmembrane regions.

sequence of HSP30 in this regard, and the protein is predicted to be localized in membranes.

The amino acid sequence deduced from the nucleotide sequence of FDD123 was similar to that of HSP30 (31.7% identity for the entire amino acid sequence) [29], and to that of YRO2 of *S. cerevisiae* (34.1% identity) [30]. Alignment of the predicted amino acid sequence of FDD123 with those of HSP30 and YRO2 revealed strong conservation of domains that are conserved among the small HSPs from *S. cerevisiae* (Fig. 3). By contrast, no significant identity scores were obtained when FDD123 was compared with the small HSPs from *Neurospora crassa* [31], *Aspergillus nidulans* [32], and *Drosophila melanogaster* [33], respectively. It seems that small HSPs form a heterogeneous group. Sequence analysis of the longest clone, DD135, derived from DD13 revealed that the deduced amino acid sequence was similar to that of the carboxy-terminal half of the ENO protein from *Xenopus laevis* [34] (92% similarity). It is likely that DD135 was actually a truncated cDNA for ENO that lacked the 5′-terminal region (data not shown).

Analysis by RT-PCR of total RNA from *C. versicolor* cells that has been grown in LN medium plus 0.5% DMF (control) or that has been exposed to 100 μM PCP plus DMF was performed with primers specific for the FDD123 and DD135 cDNA sequences. The level of FDD123 mRNA appeared to increase approximately 9.7-fold at 4 h after the start of treatment with PCP (Fig. 4). Considering the level of FDD123 mRNA detected under control conditions, we can surmise that the gene is expressed at a very low constitutive level or the DMF in control cultures acted as a weak chemical stress. Constitutive expression has been recognized for the genes of many major heat shock proteins, and such stress proteins are often essential for cell growth [35,36]. Although DD135 was expressed constitutively in control cultures, the level of the mRNA increased 1.7-fold in the presence of PCP after 4 h.

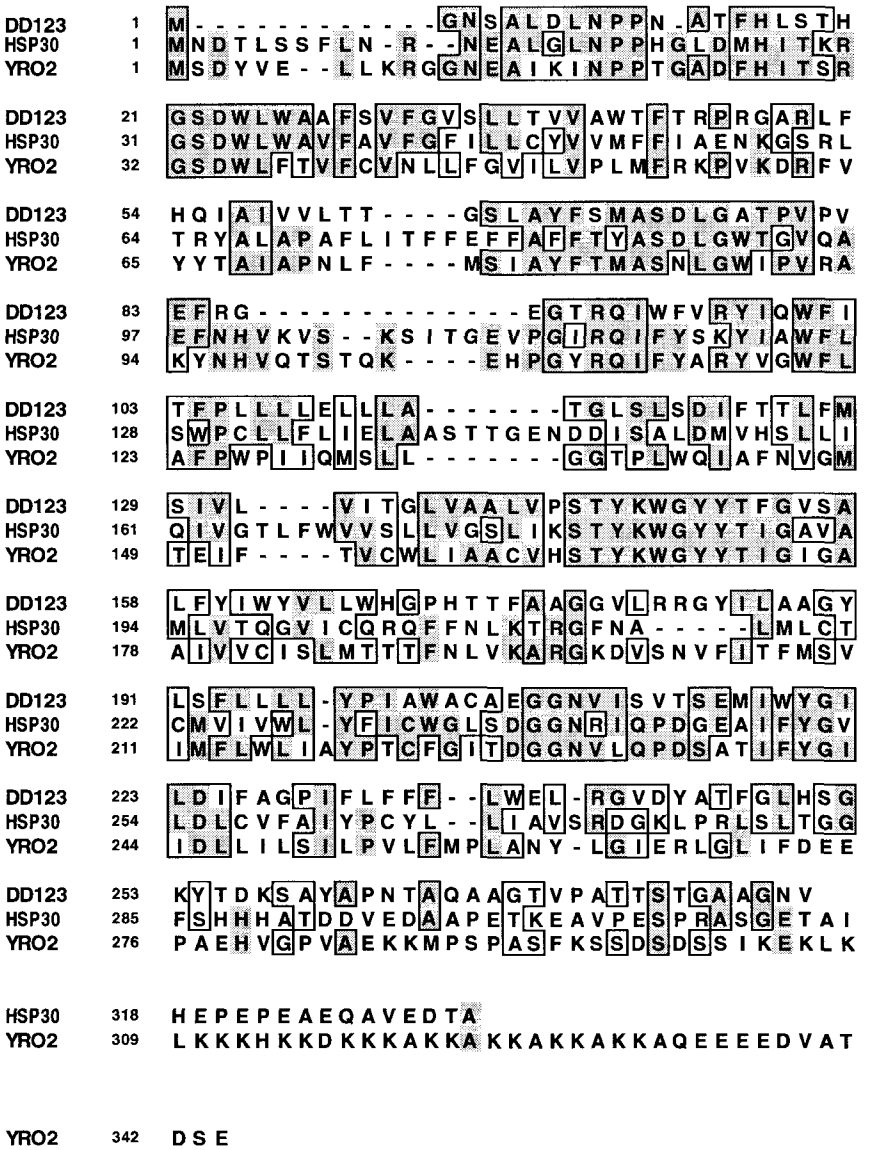


Fig. 3. Alignment of the amino acid sequence encoded by FDD123 (DD123) cDNA derived from *C. versicolor* and the sequences of HSP30 from *S. cerevisiae*, and YRO2 protein from *S. cerevisiae*. The sequences were aligned with the Clustal program. Amino acids identical to those encoded by FDD123 of *C. versicolor* are shown in shaded areas. Similar amino acids are shown in boxes.

Our findings suggest that the FDD123 gene is a member of the family of genes for HSPs. The general function of HSPs in cells is that of molecular chaperones [37,38]. Mammalian HSP27 has been identified as a molecular chaperone and an inhibitor of the polymerization of actin [39]. Its localization in cell membranes, as predicted from its amino acid sequence, suggests that FDD123 might be involved in an adaptive response that allows the maintenance of membrane integrity. The gene for enolase (DD135) was expressed constitutively in *C. versicolor*, and the mRNA accumulated at only a slightly increased level in PCP-stressed cells. Enolase, which is a glycolytic enzyme, might have an isoform-specific role in the stress response of *C. versicolor*, by analogy to observation in *S. cerevisiae* [40] and in mammalian cells[41]. We are now isolating a full-length cDNA for ENO and genomic clones for HSP, as well as for ENO. Such efforts should help us to clarify the physiological roles and the mechanisms for the chemical stress-induced expression of these gene.

References

[1] Tien, M. and Kirk, T.K. (1983) Science 221, 661–663.
[2] Kirk, T.K. and Farrell, R.L. (1987) Annu. Rev. Microbiol. 41, 465–505.
[3] Morohoshi, N. and Haraguchi, T. (1987) Mokuzai Gakkaishi 33, 143–150.
[4] Kawai, S., Umezawa, T., Shimada, M. and Higuchi, T. (1988) FEBS Lett. 236, 309–311.
[5] Gold, M.H., Wariishi, H. and Valli, K. (1989) ACS Symp. Ser. 389, 127–140.
[6] Wariishi, H., Valli, K. and Gold, M.H. (1991) Biochem. Biophys. Res. Commun. 176, 269–275.
[7] Iimura, Y., Katayama, Y., Kawai, S. and Morohoshi, N. (1995) Biosci. Biotech. Biochem. 59, 903–905.

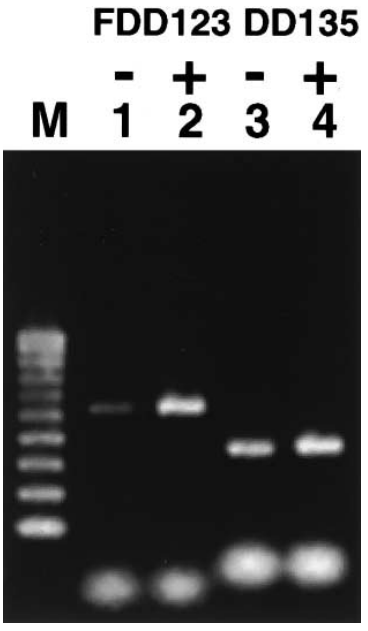


Fig. 4. Analysis by RT-PCR of transcription in *C. versicolor* upon exposure to chemical stress. Results of RT-PCR with: lanes 1 and 3, RNA from cells incubated with DMF and without PCP; and lanes 2 and 4, RNA from cells incubated with DMF and PCP. For samples in lanes 1 and 2, PCR of cDNAs was performed with the primers specific for FDD123 cDNA; for samples in lanes 3 and 4, PCR of cDNAs was performed with the primers specific for DD135 cDNA. Lane M was loaded with the 100 bp ladder of DNA (Pharmacia Biotech) for molecular weight marker.

[8] Eriksson, K.-E. and Kolar, M.-C. (1985) Environ. Sci. Technol. 19, 1086–1089.
[9] Bumpus, J.A. and Aust, S.D. (1987) Bioessays 6, 166–170.
[10] Hammel, K.E. (1989) Enzyme Microb. Technol. 11, 776–777.
[11] Valli, K. and Gold, M.H. (1991) J. Bacteriol. 173, 345–352.
[12] Iimura, Y., Hartikainen, P. and Tatsumi, K. (1996) Appl. Microbiol. Biotechnol. 45, 434–439.
[13] Pribnow, D., Mayfield, M.B., Nipper, V.J., Brown, J.A. and Gold, M.H. (1989) J. Biol. Chem. 264, 5036–5040.
[14] Adrawis, A., Pease, E.A., Kuan, I.C., Holzbaur, E. and Tien, M. (1989) Biochem. Biophys. Res. Commun. 162, 673–680.
[15] Kojima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaino, M. and Kita, Y. (1990) J. Biol. Chem. 265, 15224–15230.
[16] Gaskell, J., Stewart, P., Kersten, P.J., Covert, S.F., Reiser, J. and Cullen, D. (1994) Bio/Technology 12, 1372–1375.
[17] Yaver, D.S., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K. and Dalboge, H. (1996) Appl. Environ. Microbiol. 62, 834–841.
[18] Stewart, P., Kersten, P., Vanden Wymelenberg, A., Gaskell, J. and Cullen, D. (1992) J. Bacteriol. 174, 5036–5042.
[19] Boominathan, K., D'Souza, T.M., Naidu, P.S., Dosoretz, C. and Reddy, C.A. (1993) Appl. Environ. Microbiol. 59, 3946–3950.
[20] Gold, M.H. and Alic, M. (1993) Microbiol. Rev. 57, 605–622.
[21] Bogan, B.W., Schoenike, B., Lamar, R.T. and Cullen, D. (1996) Appl. Environ. Microbiol. 62, 3697–3703.
[22] Li, D., Alic, M., Brown, J.A. and Gold, M.H. (1995) Appl. Environ. Microbiol. 61, 341–345.
[23] Johnsson, L. and Nyman, P.O. (1994) Biochim. Biophys. Acta 1218, 408–412.
[24] Johansson, T. and Nyman, P.O. (1996) Gene 170, 31–38.
[25] Liang, P. and Pardee, A.B. (1992) Science 257, 967–971.
[26] Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E. and Farrell, R.L. (1986) Enzyme Microb. Technol. 8, 27–32.
[27] Ito, T., Kito, K., Adati, N., Mitsui, Y., Hagiwara, H. and Sakaki, Y. (1994) FEBS Lett. 351, 231–236.
[28] Forhman, M.A. (1993) Methods Enzymol. 218, 340–356.
[29] Regnacq, M. and Boucherie, H. (1993) Curr. Genet. 23, 435–442.
[30] Aljinovic, G. and Pohl, T.M. (1995) Yeast 11, 475–479.
[31] Plesofsky-Vig, N. and Brambl, R. (1990) J. Biol. Chem. 265, 15432–15440.
[32] Kusakabe, T., Koga, K. and Sugimoto, Y. (1994) Biochim. Biophys. Acta 1219, 555–558.
[33] Southgate, R., Ayme, A. and Voellmy, R. (1983) J. Mol. Biol. 165, 35–57.
[34] Segil, N., Shrutkowski, A., Dworkin, M.B. and Dworkin-Rastl, E. (1988) Biochem. J. 251, 31–39.
[35] Straus, D.B., Walter, W.A. and Gross, C.A. (1987) Nature 329, 348–351.
[36] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[37] Rothman, J.E. (1989) Cell 59, 591–601.
[38] Beckmann, R.P., Mizzen, A.L. and Welch, W.J. (1990) Science 248, 850–854.
[39] Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M. and Geiger, B. (1991) J. Cell Biol. 114, 255–261.
[40] Iida, H. and Yahara, I. (1985) Nature 315, 688–690.
[41] Aaronson, R.M., Graven, K.K., Tucci, M., McDonald, R.J. and Farber, H.W. (1995) J. Biol. Chem. 270, 27752–27757.