α_1-Antitrypsin can increase insulin-induced mitogenesis in various fibroblast and epithelial cell lines

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Received 24 February 2000; received in revised form 3 April 2000

Edited by Veli-Pekka Lehto

Abstract α_1-Antitrypsin (AT), the archetypal member of the superfamily of serine proteinase inhibitors, inhibits leukocyte elastase activity and thereby can prevent lung damage. Here we show that in fibroblasts from human fetal lung and mouse embryo as well as in certain epithelial cells AT can also enhance the stimulatory effects of insulin on DNA synthesis and cell proliferation. Warming of AT at a moderate (41°C) temperature for a longer time (21 h) or at a higher (65°C) temperature for 30 min before treatment increased its stimulatory effects on both DNA synthesis and activating phosphorylation of p42/p44 mitogen-activated protein kinases. The results suggest that AT may promote regeneration of damaged tissues under pathophysiological conditions which are associated with fever.

Key words: α_1-Antitrypsin; Insulin; Fibroblast; DNA synthesis; Mitogen-activated protein kinase

1. Introduction

Human α_1-antitrypsin (AT), a member of the serpin superfamily, is a highly polymorphic glycoprotein (~52 000 kDa) which is synthesized by the liver and, to a lesser extent, by mononuclear phagocytes and neutrophils [1–5]. AT functions as the principal inhibitor of neutrophil elastase in the lower respiratory tract. So far, no other function of AT has been reported. In AT deficiency, which is an autosomal recessive hereditary disorder, insufficient amounts of AT fail to prevent elastolytic destruction of the pulmonary parenchyma greatly increasing the risk of developing chronic obstructive pulmonary disease or emphysema (referenced in [6]). Interestingly, smoking and other kinds of oxidative damage to an active-site methionine residue can also greatly decrease the elastase inhibitory capacity of AT [7–9]. This may underlie the pathogenesis of pulmonary emphysema in cigarette smokers.

The AT molecule, like other serpin molecules, is made up of nine helices and three pleated sheets (β-sheets, A, B, and C), the dominant feature of the molecule being the five-stranded A sheet with the reactive center loop of the molecule arising from it. Biochemical data and X-ray crystallographic studies have shown that AT can adopt several different structures. This can occur because areas including the reactive center loop and the A β-sheet are extremely mobile allowing AT to undergo a conformational switch termed the stressed (native) to relaxed (latent) transition. The instability of AT can be enhanced by various mutations and heat; in turn, AT molecules will form polymers due to linkages developing between the reactive loop of one molecule and the β-pleated sheet of the next molecule [10–17]. If such aggregation occurs inside hepatocytes, this will lead to plasma deficiency of AT as well as lung and liver damage (reviewed in [18]).

We recently purified placental alkaline phosphatase (PALP) from a commercial preparation to study its effect on mitogenesis [19]. During this work we noticed the presence of a major contaminant in the starting material which was mitogenically active under specific conditions. After complete purification, this protein was identified as AT, which made us interested in determining its effects on DNA synthesis, cell proliferation, and the accompanying mechanisms. We now report that after warming at 41°C and 65°C for 21 h and 30 min, respectively, AT can enhance the stimulatory effects of insulin on DNA synthesis and cell proliferation in fibroblasts, isolated from either a second-trimester fetus or a mouse embryo, and in various other cell lines of epithelial origin.

2. Materials and methods

2.1. Materials

Human PALP, AT and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were bought from Sigma. Insulin was purchased from Boehringer Mannheim. [*methyl*-^{3}H]Thymidine (500 mCi/mmol) and [γ-^{32}P]ATP (60 Ci/mmol) were from Dupont NEN. The p42/p44 as well as p38 mitogen-activated protein (MAP) kinase kits were bought from New England Biolabs. Tissue culture reagents, including Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum, were bought from Gibco-BRL.

2.2. Cell culture

NIH 3T3 fibroblasts, the HTB-157 fetus (second trimester) lung fibroblast-like cells, and MRC-9 lung fibroblasts, each bought from American Type Culture Collection, were cultured in 10% fetal calf serum-containing DMEM as indicated in [20]. The sources for mouse epidermal JB6 cells and culture conditions were as indicated in [21].

2.3. Purification of human AT

A partially purified human PALP preparation from Sigma was first further purified by successive concanavalin A-Sepharose and Q-Sepharose chromatography as described by Chang et al. [22]. Finally, the Q-Sepharose fraction, which still contained PALP in addition to AT, was purified to homogeneity by *t*-butyl HIC chromatography [22]. The 5 ml bed volume *t*-butyl HIC cartridge was connected to a Pharmacia FPLC system. The purity was confirmed by SDS–PAGE using Coomassie blue stain. Sequence analysis which identified the purified protein as AT was performed independently by the Mayo Clinic Protein Core Facility (Rochester, MN, USA).

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Abbreviations: AT, α_1-antitrypsin; PALP, placental alkaline phosphatase; MAP, mitogen-activated protein; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

PII: S0014-5793(00)01492-7

2.4. Determination of DNA synthesis

Fibroblasts were grown in 12 well tissue culture dishes to about 40–50% confluence in 10% fetal calf serum-containing DMEM, washed, and then incubated in serum-free medium for 27 h. The cells were washed and then treated for 17 h in serum-free medium with AT and insulin, followed by incubation for 1 h in the presence of [^{3}H]thymidine (1 mCi/well). The cells were washed twice with phosphate-buffered saline, then four times with 5% trichloroacetic acid, and finally twice with absolute ethanol. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide and the ^{3}H activity was counted.

2.5. MAP kinase phosphorylation assay

Serum-starved (24 h) NIH 3T3 fibroblasts, grown in 35 mm diameter dishes to 70–80% confluence, were incubated for 10 min with AT in the absence or presence of 500 nM insulin. Samples for Western immunoblot analysis were prepared as described earlier [23]. Phospho-specific antibodies (New England Biolabs) recognizing specific, activating, phosphorylated sites in p42/p44 as well as p38 MAP kinases were used to quantitate the phosphorylated/activated forms of these enzymes. The relative changes in the intensity of phosphorylation were determined by a Storm 840 phosphorimager (Molecular Dynamics).

2.6. Cell proliferation assay

Changes in the number of viable cells in serum-starved (24 h) NIH 3T3 cultures (cells seeded in 96 well plates at 1000 cell per well) upon treatment with AT and insulin were determined by the MTT method [24] as described previously in detail [25]. A Labsystem Multiskan MS microplate reader was used to read absorbance and to analyze data [25].

3. Results

Sigma PALP was used as the starting material to obtain a completely purified protein fraction which, after separation by gel electrophoresis, contained only one band (Fig. 1, lane 5). Sequence analysis revealed that this protein is identical to AT. We do not know, as yet, whether AT in the Sigma preparation originated from contaminating blood, the placenta itself, or both. Eleven different preparations of AT were used during this study with no major differences in their effects on mitogenesis and signal transduction. Each effect of AT reported here was reproduced by commercial (Sigma) AT, except that in the latter case about three times more protein was required to achieve the same effect. This discrepancy reflects that in the Sigma AT preparation impurities represent about 60–70% of the total protein. Since a contaminant in our preparations was not detected, and the Sigma AT was prepared from blood with a different method, it is extremely unlikely that the similar results obtained with the Sigma and our AT preparations were due to a common contaminant.

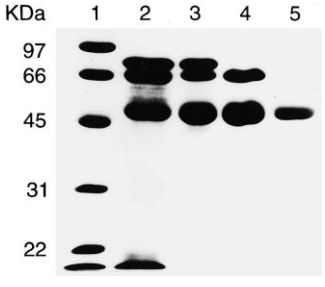

Fig. 1. Purification of AT. The Sigma PALP preparation (lane 2) was used as the starting material. The purification steps included successive concanavalin A-Sepharose, (lane 3), Q-Sepharose (lane 4), and *t*-butyl HIC chromatography (lane 5), as described in Section 2. The purified ~52 kDa AT is shown in lane 5, while the molecular mass standards are indicated in lane 1.

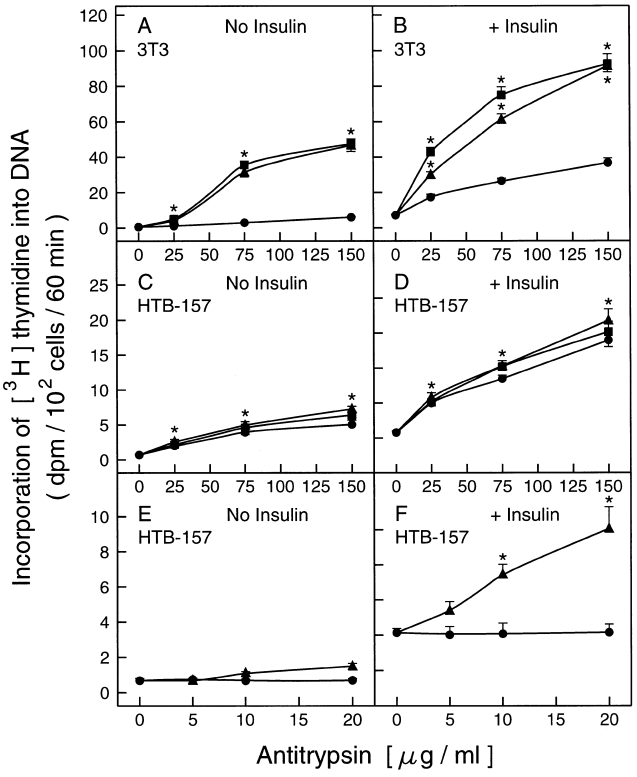

Fig. 2. Effects of AT on DNA synthesis in mouse and human fetal lung fibroblasts. Serum-starved (27 h) mouse embryo NIH 3T3 (A,B) and human HTB-157 lung fibroblasts (C–F) were incubated for 18 h, with [^{3}H]thymidine being added to cells for the last hour of treatment, in the absence (A,C,E) or presence (B,D,F) of 500 nM insulin, with the indicated concentrations of AT; AT was untreated (●) or pre-warmed at 41°C for 21 h (▲), or at 65°C for 30 min (■) immediately before use. The data represent the mean ± S.E.M. of three experiments each performed in triplicate. *Significantly ($P < 0.01$–0.05) different from the corresponding value in the absence of AT (Student's *t*-test).

In serum-starved mouse NIH 3T3 fibroblasts, purified AT (used in the 25–150 μg/ml range) alone significantly stimulated DNA synthesis only if it was warmed for 21 h at 41°C or for 30 min at 65°C prior to treatments (Fig. 2A). While even untreated AT was able to slightly increase the small effect of insulin, warming of AT at either 41°C or 65°C clearly enhanced its potentiating effects (Fig. 2B). Importantly, heat-treated AT retained its ability to increase insulin-stimulated DNA synthesis for at least 3 days when stored at 4°C.

In serum-starved lung fibroblasts originally derived from a 4 month old fetus, both untreated and heat-treated AT, used at 25–150 μg/ml concentrations, stimulated DNA synthesis in the absence of insulin (Fig. 2C) as well as enhancing the effect of insulin (Fig. 2D). However, at lower (5–20 μg/ml) concentrations AT alone had no effects even after heat treatment (Fig. 2E), while in the presence of insulin warming of AT was required to observe potentiating effects (Fig. 2F).

Serum starvation usually elicits rapid apoptotic cell death in most fibroblast lines. However, incubation of NIH 3T3 fibroblasts in serum-free medium for 2 days did not significantly

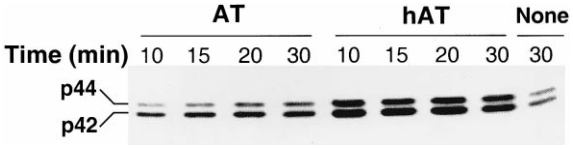

Fig. 3. Western blot analysis of stimulatory effects of AT on activating phosphorylation p42/p44 MAP kinases. Serum-starved NIH 3T3 fibroblasts were treated with 75 μg/ml of untreated AT (AT) or pre-warmed (65°C, 30 min) AT (hAT) for up to 30 min as indicated. hAT had similar effects on MAP kinase phosphorylation in two other experiments.

reduce the number of viable cells as determined by the MTT colorimetric assay and verified by direct cell counting [19]. During 2 days of incubation in serum-free medium, separate exposure of NIH 3T3 cells to insulin and pre-heated (65°C, 30 min) AT enhanced cell numbers only slightly (~1.3- and 1.15-fold increases respectively), while in combination they had an about 1.6-fold (±0.18, S.E.M.) stimulatory effect (data not shown). Thus, insulin and heat-treated AT in combination increased not only DNA synthesis but also cell proliferation.

In serum-starved NIH 3T3 and fetal fibroblasts, the combined effects of insulin and heat-treated (65°C, 30 min) AT on DNA synthesis were inhibited 46–55% by 50 μM PD 98059, a MAP kinase kinase inhibitor [26], 80–86% by rapamycin (10 nM), a specific inhibitor of the actions of p70 S6 kinase [27], and 31–39% by wortmannin (200 nM), an inhibitor of phosphatidylinositol 3′-kinase [28]. These data suggested that AT and insulin may stimulate both the p44/p44 MAP kinase- and the p70 S6 kinase-dependent mitogenic pathways.

Considering the well established role of activating phosphorylation of p42/p44 MAP kinases in promoting mitogenesis in fibroblasts [29] as well as the inhibitory effects of PD 98059 on insulin+AT-induced DNA synthesis (discussed above), these enzymes may be involved in the mediation of mitogenic insulin and AT effects. In agreement with this possibility, heat-treated (65°C, 30 min) AT (75 μg/ml) greatly enhanced activating phosphorylation of both p42 and p44 MAP kinases (Fig. 3). In comparison, untreated AT had much smaller, although still well detectable, effects on MAP kinase phosphorylation (Fig. 3). In NIH 3T3 cells, insulin had only very small effects on MAP kinase phosphorylation [21], and it did not detectably alter the effects of AT (not shown).

It has been reported that in NIH 3T3 cells serum deprivation for 20 h results in increased activating phosphorylation of p38 MAP kinase [30], an enzyme which appears to be part of the signaling cascade leading to mitotic arrest and apoptosis

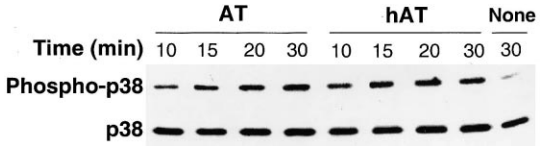

Fig. 4. Western blot analysis of stimulatory effects of AT on activating phosphorylation of p38 MAP kinase. Serum-starved NIH 3T3 fibroblasts were treated with 75 μg/ml of untreated AT or pre-warmed AT (hAT) for up to 30 min. The upper and lower panels show the phosphorylated and non-phosphorylated forms of p38 MAP kinase, respectively. hAT had similar effects on p38 MAP phosphorylation in two other experiments.

[30–33]. This suggested to us that part of the AT action on mitogenesis may involve inhibition of p38 MAP kinase phosphorylation. However, in our hands, serum starvation for 24 h resulted in barely detectable phosphorylation of p38 MAP kinase which was actually enhanced by both untreated and heat-treated ATP over a 30 min incubation period (Fig. 4). However, phosphorylation of p38 MAP kinase was significantly increased after an incubation for 3 days in serum-free medium, and this was partially inhibited by both untreated and heat-treated AT (data not shown).

Since the primary target of AT may be epithelial cells, we also examined the effects of AT in serum-starved LA-4 lung epithelial (adenoma) cells. Although these, like other transformed cells, are generally less sensitive to actions of growth factors, insulin still stimulated DNA synthesis about 1.8-fold. Furthermore, in LA-4 cells heat-treated (41°C, 22 h) and untreated AT, used in the range of 20–150 μg/ml, further enhanced the effects of insulin 1.4–1.9- and 1.2–1.5-fold, respectively (data not shown). These data suggest that AT may similarly enhance insulin-stimulated mitogenesis in lung fibroblasts and lung epithelial cells. In addition to the above cell lines, heat-treated AT (150 μg/ml) also increased insulin-stimulated DNA synthesis in MRC-9 lung fibroblasts and JB6 (clone-41) mouse epidermal cells at least two-fold. In contrast, AT had no detectable effects on DNA synthesis in MRC-5 lung fibroblasts, A_7r5 endothelial cells, and CCD 1058 and CCD 944 normal human fibroblast lines derived from adult skin, although these cell lines responded to insulin (data not shown). Clearly, AT is not a universal enhancer of insulin action on mitogenesis.

4. Discussion

The major observation of this work is that in selected cell lines of both mesenchymal and epithelial origin, but not in all insulin-sensitive cell lines examined, highly purified AT can enhance DNA synthesis and cell proliferation particularly if insulin is also present. Pre-warming of AT even at a moderate (41°C) temperature increased the effects of AT. Since insulin is a progression factor, it is reasonable to assume that AT acts by stimulating re-entrance of quiescent cells into the cell cycle. However, AT is clearly an incomplete mitogen because in the absence of a progression factor it has smaller effects on DNA synthesis.

Heat-treated, and to a lesser extent untreated, AT also stimulated activating phosphorylation of p42/p44 MAP kinases, suggesting that these enzymes mediate, at least in part, the mitogenic effects of AT. In addition, in serum-starved (24 h) NIH 3T3 cells both untreated and heat-treated AT also enhanced activating phosphorylation of p38 MAP kinase, an enzyme whose activation is usually associated with apoptotic cell death [30–33]. However, after longer incubations AT partially inhibited the serum withdrawal-induced increase in p38 MAP kinase phosphorylation. Since the survival factor Gas6 has similar biphasic effects on p38 MAP kinase phosphorylation [30], AT may also promote cell survival. This latter possibility remains to be determined.

Since both heat-treated and untreated AT had stimulatory effects on both DNA synthesis and MAP kinase phosphorylation, it is reasonable to assume that their effects involved similar mechanisms mediated by the same membrane site. The primary membrane target of AT actions remains to be deter-

mined. Similarly, it is presently unclear how an even moderate heat treatment can increase the effects of AT on DNA synthesis. However, it is known that exposure to heat can cause various changes in the structure of AT. First, since the areas including the reactive center loop and the A β-sheet are extremely mobile, this allows AT to undergo a conformational switch from the stressed (native) to the relaxed (latent) state. In the latent form (which does not interact with elastase) the center loop is stably incorporated into the A sheet of the same molecule. In addition, AT molecules can form polymers of various length via insertion of the reactive center loop of one molecule into a β-pleated sheet of a second [10–17]. Since after treatment at 65°C for 30 min practically all AT molecules are found in polymers [34], and in NIH 3T3 cells AT is mitogenically more active after heating at this temperature, it is reasonable to assume that AT polymers are more active on mitogenesis than AT monomers. However, to prove this point further, it will be necessary to rigorously compare the rate of AT polymerization to changes in mitogenesis after exposing AT to various temperatures for varying lengths of time.

The finding that even moderate heating of AT increases its mitogenic activity may be important. Depending on the conditions of the equilibrium among various conformational states of AT, at normal body temperature a minor fraction of AT could already be in a mitogenically active conformation. However, various infections, which usually increase both body temperature and expression of AT, and other physiological conditions which induce fever, could increase the concentration of the mitogenically active form several-fold. Such heat regulation may ensure that AT will enhance mitogenesis only when it is really needed, i.e. following tissue damage. Considering that the optimal effects on mitogenesis require only relatively small amounts of AT, and that the normal concentration of AT in the circulation is about 2–2.5 mg/ml, it is likely that even during prolonged fever sufficient numbers of AT molecules will still be in the stressed state capable of binding elastase. Overall, the data presented here suggest that physiological conditions which result in fever may also endow a fraction of serum AT molecules with mitogenic activity without compromising the ability of most AT molecules to neutralize elastase activity.

In summary, our work suggests that AT, in addition to its well established role of neutralizing elastase activity, may also enhance mitogenesis in certain tissues. The primary membrane target of AT remains to be elucidated. Since a modest increase above normal body temperature appears to be both required and sufficient to induce a mitogenically active conformation, it is very likely that AT will exert positive effects on mitogenesis only under physiological conditions which are associated with fever. This may ensure that AT acts as a mitogen or promitogen only when a damaged tissue needs to be repaired. Since heat-treated AT retains its mitogenic effects for several days, activated AT may find a use for therapeutic purposes.

Acknowledgements: This work was supported by SOTA TEC Fund 343-6027.

References

[1] Fagerhol, M.K. and Cox, D.W. (1981) Adv. Hum. Genet. 11, 1–62.
[2] Carrell, R.W., Jeppsson, J.-O., Laurell, C.-B., Brennan, S.O., Owen, M.C., Vaughan, L. and Boswell, D.R. (1982) Nature 298, 329–334.
[3] Loebermann, H., Tokuoka, R., Deisenhofer, J. and Huber, R. (1984) J. Mol. Biol. 177, 531–556.
[4] Stein, P.E. and Carrell, R.W. (1995) Nature Struct. Biol. 2, 96–113.
[5] Worrall, D.M., Blacque, O.E. and Barnes, R.C. (1999) Biochem. Soc. Trans. 27, 746–750.
[6] Curiel, D.T., Vogelmeier, C., Hubbard, R.C., Stier, L.E. and Crystal, R.G. (1990) Mol. Cell. Biol. 10, 47–56.
[7] Janoff, A., Carp, H. and Lee, D.K. (1979) Science 206, 1313–1314.
[8] Gadek, J.E., Fells, G.A. and Crystal, R.G. (1979) Science 206, 1315–1316.
[9] Johnson, D. and Travis, J. (1979) J. Biol. Chem. 254, 4022–4026.
[10] Lomas, D.A., Finch, J.T., Sayama, K., Nukiwa, T. and Carrell, R.W. (1993) J. Biol. Chem. 268, 15333–15335.
[11] Kwon, K.-S., Kim, J., Shin, H.S. and Yu, M.-H. (1994) J. Biol. Chem. 269, 9627–9631.
[12] Sidhar, S.K., Lomas, D.A., Carrell, R.W. and Foreman, R.C. (1995) J. Biol. Chem. 270, 8393–8396.
[13] Hopkins, P.C.R. and Stone, S.R. (1995) Biochemistry 34, 15872–15879.
[14] Lomas, D.A., Elliott, P.R., Sidhar, S.K., Foreman, R.C., Finch, J.T., Cox, D.W., Whisstock, J.C. and Carrell, R.W. (1995) J. Biol. Chem. 270, 16864–16870.
[15] Bottomley, S.P. and Chang, W.-S.W. (1997) Biochem. Biophys. Res. Commun. 241, 264–269.
[16] James, E.L. and Bottomley, S.P. (1998) Arch. Biochem. Biophys. 356, 296–300.
[17] James, E.L., Whisstock, J.C., Gore, M.G. and Bottomley, S.P. (1999) J. Biol. Chem. 274, 9482–9488.
[18] Carrell, R.W., Lomas, D.A., Sidhar, S. and Foreman, R. (1996) Chest 110, 243S–247S.
[19] She, Q.-B., Mukherjee, J.J., Huang, J.-S. and Kiss, Z. (2000) FEBS Lett. 469, 163–167.
[20] Kiss, Z., Rapp, U.R., Pettit, G.R. and Anderson, W.B. (1991) Biochem. J. 276, 505–509.
[21] Kiss, Z., Anderson, W.H. and Mukherjee, J.J. (1998) Biochem. J. 330, 819–826.
[22] Chang, T.C., Huang, S.M., Huang, T.M. and Chang, G.G. (1992) Eur. J. Biochem. 209, 241–247.
[23] Kiss, Z. and Anderson, W.H. (1994) Biochem. J. 300, 751–756.
[24] Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. and Mitchell, J.B. (1987) Cancer Res. 47, 936–942.
[25] Malewicz, B., Mukherjee, J.J., Crilly, K.S., Baumann, W.J. and Kiss, Z. (1998) Eur. J. Biochem. 253, 10–19.
[26] Pang, L., Sawada, T., Decker, S.J. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 13585–13588.
[27] Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Cell 68, 1227–1236.
[28] Arcaro, A. and Wyman, M.P. (1993) Biochem. J. 296, 297–301.
[29] Pagés, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Méloche, S. and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. USA 90, 8319–8323.
[30] Goruppi, S., Ruaro, E., Varnum, B. and Schneider, C. (1999) Oncogene 18, 4224–4236.
[31] Xia, Z., Dickens, M., Raigeaud, J., Davis, R.J. and Greenberg, M.E. (1995) Science 270, 1326–1331.
[32] Kummer, J.L., Rao, P.K. and Heidenreich, K.A. (1997) J. Biol. Chem. 272, 20490–20494.
[33] Takenaka, K., Moriguchi, T. and Nishida, E. (1998) Science 280, 599–602.
[34] Lomas, D.A., Elliott, P.R., Chang, W.S.W., Wardell, M.R. and Carrell, R.W. (1995) J. Biol. Chem. 270, 5282–5288.