Species specificity of the cytokine function of phosphoglucose isomerase

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Abstract Phosphoglucose isomerase (PGI) is a cytosolic glycolytic enzyme that also functions as an extracellular cytokine (neuroleukin/autocrine motility factor (AMF)/maturation factor). Contrary to mammalian PGI, bacterial PGI was not internalized by the PGI/AMF receptor (gp78/AMF-R) and neither bacterial nor yeast PGI competed with mammalian PGI for receptor binding and internalization. Furthermore, while the bacterial, yeast and mammalian preparations all exhibited isomerase activity, only mammalian PGI stimulated the motility of NIH-3T3 fibroblasts. The conserved active site of PGI is therefore not sufficient for receptor binding and cytokine activity of PGI. However, synthetic peptides corresponding to distinct peripheral mammalian PGI sequences did not inhibit internalization of mammalian PGI. Our data therefore argue that the cytokine activity of PGI is specific to mammalian PGI but cannot exclude the possibility that the receptor binding motif of PGI is complex and includes elements within and without the active site. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Phosphoglucose isomerase; Autocrine motility factor receptor; Cell motility; Cytokine; Endocytosis; Glycolysis

1. Introduction

Phosphoglucose isomerase (PGI, EC 5.3.1.9) is an essential cytosolic enzyme expressed in all tissues that catalyzes the conversion of D-glucose 6-phosphate and D-fructose 6-phosphate. It plays an essential role in glycolysis and gluconeogenesis. An extracellular cytokine function for PGI has also been identified and the PGI polypeptide sequence is identical to that of autocrine motility factor (AMF), neuroleukin and maturation factor [1–4]. PGI (or neuroleukin) increases the survival of cultured sensory neurons and is secreted by lectin-stimulated T-cells and induces maturation of B-cells into antibody secreting cells [5,6]. As maturation factor, PGI has been shown to induce the differentiation of human myeloid cells into monocytes [4]. PGI has also been shown to be involved in osteoblast differentiation during mineralization [7]. As AMF, PGI was identified as a cytokine secreted by tumor cells whose expression is linked to tumor cell invasion and metastasis [1,8].

AMF stimulates cell motility and growth by binding to its cell surface receptor, a glycoprotein of 78 kDa, referred to as gp78 or AMF receptor (AMF-R) [9–11]. AMF-R is a seven-transmembrane domain, pertussis toxin-sensitive G protein-coupled receptor that has recently been identified as an E3 ubiquitin protein ligase [12,13]. AMF-R expression is associated with tumor motility and metastasis and significantly increased in neoplastic tissue [14]. AMF-R is expressed in cerebellar and hippocampal neurons and its activation may play a role in neuronal plasticity during development and in memory and learning [15,16].

The fact that a glycolytic enzyme also exhibits cytokine activity is intriguing and raises questions as to the relationship between its enzymatic and cytokine activities. The crystal structures of mammalian (rabbit, pig, human) and bacterial (*Bacillus stearothermophilus*) PGI have been determined and show that the active site of the functional homodimeric enzyme is conserved evolutionarily [17–22]. Inhibitors of PGI activity prevent the motility stimulating activity of PGI [1,7,23]. Furthermore, the bacterial form of the enzyme has been reported to stimulate the motility of CT-26 mouse colon tumor cells and this motility stimulation is sensitive to inhibition by the PGI inhibitor 5-phosphoarabinoate [17,19]. These data support a role for the evolutionarily conserved enzymatic site in receptor binding of PGI.

AMF-R internalizes its ligand via two pathways, a clathrin-mediated pathway to multivesicular bodies (MVBs) and a caveolae-mediated pathway to the endoplasmic reticulum [24–26]. In order to address the nature of the interaction of PGI with its receptor, we have used the clathrin-dependent endocytosis of AMF-R as an assay for receptor binding [25]. We report that bacterial PGI is not internalized by NIH-3T3 cells and that neither bacterial nor yeast PGI compete with mammalian PGI for receptor binding and internalization. Furthermore, neither the bacterial nor yeast forms of the enzyme are able to stimulate the motility of NIH-3T3 cells. These results demonstrate that the cytokine activity of PGI is specific to mammalian PGI and argue that the PGI enzymatic site is not sufficient for receptor binding.

2. Materials and methods

2.1. Cells and reagents

NIH-3T3 fibroblasts were as previously described [25]. Bacterial, yeast and mammalian PGI were obtained from Sigma (Oakville, ON, Canada). Bacterial and rabbit PGI were biotinylated with NHS-LC-biotin (Pierce, Rockford, IL, USA) or rabbit PGI conjugated to Alexa-568 (Molecular Probes, Eugene, OR, USA) as per the manufacturers' instructions. Horseradish peroxidase and Texas Red-conjugated streptavidin were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Synthetic peptides

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Abbreviations: AMF, autocrine motility factor; AMF-R, autocrine motility factor receptor; MVB, multivesicular body; PGI, phosphoglucose isomerase

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corresponding to amino acids 1–10 (AALTRNPQFQ), 362–373 (YITKSGARVDHQ), 443–460 (EARKELQAAGKSPEDLEK), and 547–557 (FIKQQRDTKLE) of the mouse PGI sequence were purchased from the NRC Biotechnology Research Institute (Montreal, QC, Canada).

2.2. Immunofluorescence labeling

Endocytosis of biotinylated or Alexa-568-conjugated PGI to MVBs in NIH-3T3 cells was performed as previously described [25]. For the competition assays, cells were pulsed for 30 min at 37°C with labeled PGI plus the indicated concentrations of unlabeled rabbit, bacteria or yeast PGI or of selected synthetic peptides. Cells were washed and fixed, and biotinylated PGI revealed as described [25] and Alexa-568-PGI visualized directly. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals, Allentown, PA, USA) and viewed with the 63× Zeiss Plan Apochromat objective, selective filters and a QImaging digital camera or with the 60× NikonPlan Apochromat objective of a Bio-Rad MRC600 confocal microscope. To quantify endocytosed biotinylated PGI, at least 10 fields per experiment were randomly selected from at least three experiments. The fluorescence intensity per cell was quantified using Northern Eclipse image analysis software (Empix Imaging, Mississauga, ON, Canada). For all values, control labeling in the absence of biotinylated PGI was subtracted.

2.3. SDS–PAGE and immunoblotting

Protein concentration was measured using the BCA protein assay (Pierce). One μg of native or biotinylated PGI was separated by SDS–PAGE and proteins revealed with Coomassie blue. Alternatively, the gel was blotted to nitrocellulose membranes (Amersham) and biotinylated PGI detected with horseradish peroxidase-conjugated streptavidin and chemiluminescence.

2.4. PGI enzymatic activity

PGI activity was assayed as previously described [27]. One ml of the reaction mixture (50 mM triethanolamine buffer, pH 8.3, 1 mM EDTA, 4 mM fructose 6-phosphate as a substrate, 0.5 mM NADP and one unit of glucose 6-phosphate dehydrogenase) was preincubated at 30°C for 5 min in a 1 cm light quartz cuvette in the sample compartment of a thermostatically regulated spectrophotometer (UV160U, UV-Visible recording spectrophotometer, Shimadzu Co.). The reaction was initiated by the addition of 80 ng of rabbit, bacteria or yeast PGI. The rate of isomerization was monitored for 10 min and determined from the linear reaction phase.

2.5. Phagokinetic track motility assay

NIH-3T3 cells were plated (2000 cells/35 mm dish) on glass coverslips coated with colloidal gold prepared as described [10]. After 24 h incubation, the cells were fixed with 3% paraformaldehyde and the phagokinetic tracks visualized using dark-field illumination with the 10× objective of a Zeiss Axiovert inverted microscope equipped with a Princeton MicroView CCD camera. The area cleared of gold particles by at least 100 cells was thresholded and measured using Northern Eclipse image analysis software (Empix Imaging).

3. Results

We have previously reported that commercial rabbit PGI/AMF is internalized via both clathrin-dependent and clathrin-independent pathways [24–26]. For this study, we have used the clathrin-dependent endocytosis of biotinylated rabbit PGI to MVBs to assess receptor binding and internalization [25]. It

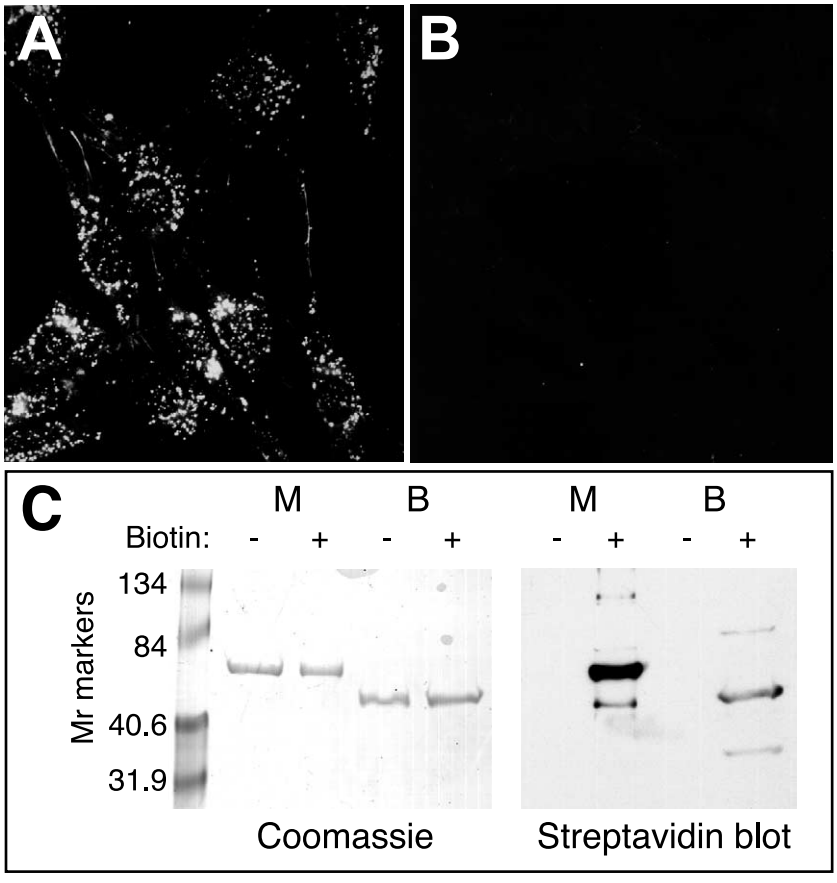

Fig. 1. Bacterial PGI is not endocytosed by NIH-3T3 cells. NIH-3T3 cells were incubated for 30 min with 100 μg/ml of mammalian biotinylated PGI (A) or bacterial biotinylated PGI (B) and after fixation, endocytosed biotinylated PGI was revealed with Texas Red streptavidin. Only mammalian PGI can be detected within intracellular MVBs. SDS–PAGE analysis (C) of 1 μg total protein revealed the presence of native (−) and biotinylated (+) mammalian (M) and bacterial (B) PGI by Coomassie blue staining (left) and by horseradish peroxidase-streptavidin labeling (right).

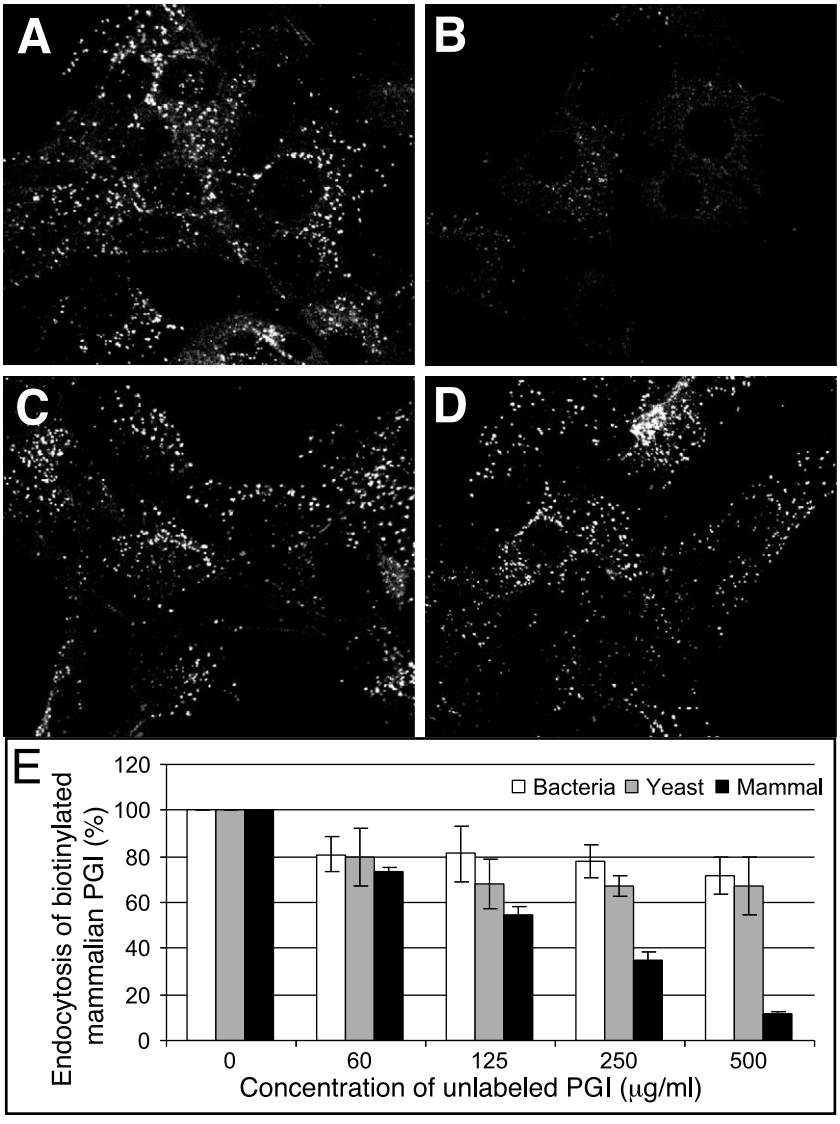

Fig. 2. Bacterial and yeast PGI do not compete with mammalian PGI for receptor binding and endocytosis. NIH-3T3 cells were incubated for 30 min with 15 μg/ml mammalian biotinylated PGI in regular medium (A) or in regular medium supplemented with 500 μg/ml mammalian (B), bacterial (C) or yeast (D) PGI and after fixation, the endocytosis of biotinylated mammalian PGI was revealed with Texas Red streptavidin. The endocytosis of biotinylated mammalian PGI in the presence of 60, 125, 250, and 500 μg/ml bacterial (white), yeast (gray), and mammalian (black) PGI was quantified (E) and is presented as the percent of endocytosis in the absence of unlabeled PGI (±S.E.M.). Reduction in the endocytosis of biotinylated mammalian PGI was only observed with increasing concentrations of native mammalian PGI.

has been reported that the bacterial form of PGI stimulates the motility of murine CT-26 cells [17,19] and we therefore undertook to determine whether bacterial PGI recognizes mammalian AMF-R and is internalized. As seen in Fig. 1A, biotinylated rabbit PGI is endocytosed to MVBs in NIH-3T3 cells after a 30 min incubation at 37°C while a similarly biotinylated bacterial PGI is not detected within the cells (Fig. 1B). The bacterial form of the enzyme migrates slightly faster than rabbit PGI and the biotinylated form of both enzymes is detected by blot with streptavidin-horseradish peroxidase (Fig. 1C).

Confirmation that receptor binding is specific for the mammalian form of the enzyme was obtained by the ability of 500 μg/ml unconjugated rabbit but not bacterial or yeast PGI to compete with the endocytosis of biotinylated rabbit PGI to MVBs (Fig. 2A–D). Increasing concentrations of unlabeled rabbit PGI progressively compete with biotinylated rabbit PGI for receptor binding and subsequent endocytosis to MVBs such that at 500 μg/ml essentially no internalized biotinylated rabbit PGI is detected (Fig. 2B,E). The addition of an excess of the yeast and bacterial forms of PGI partially ($<30\%$) reduced the internalization of rabbit PGI (Fig. 2E). The extent of the reduction did not increase with PGI concentration indicating that they are not competing directly with mammalian PGI for binding to the cell surface PGI receptor (gp78/AMF-R).

The isomerase activity of the bacterial, yeast and rabbit enzymes was measured and all three commercial preparations used exhibited significant levels of enzyme activity (Fig. 3A). Indeed, isomerase activity of the bacterial and yeast preparations was superior to that of the rabbit enzyme. Addition of 1 μg/ml of rabbit PGI to NIH-3T3 cells plated on colloidal

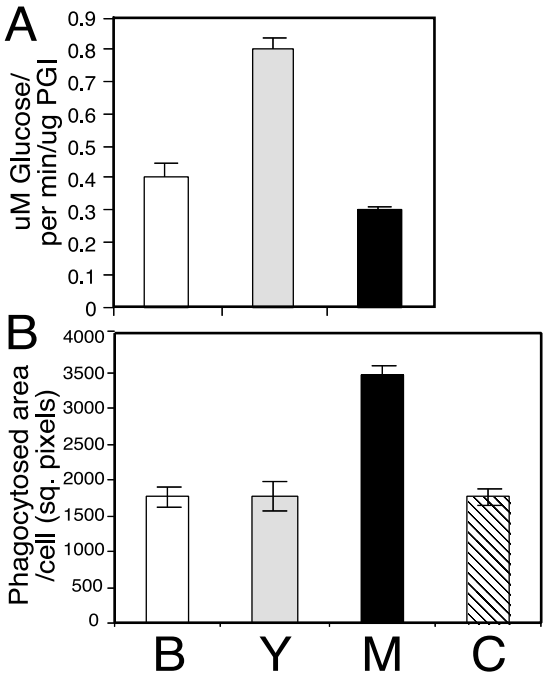

Fig. 3. Bacterial and yeast PGI preparations exhibit isomerase activity but do not stimulate the motility of NIH-3T3 fibroblasts. (A) The isomerase activity of bacterial (B, white), yeast (Y, gray), and mammalian (M, black) PGI was measured and the three preparations exhibited significant activity. Minimal activity was detected in the absence of PGI and was subtracted from the presented values. (B) Using a phagokinetic track motility assay, the ability of 1 μg/ml bacterial (B, white), yeast (Y, gray), and mammalian (M, black) PGI to stimulate the motility of NIH-3T3 cells relative to basal control motility in the absence of added PGI (C, striped) was measured. Increased motility was only detected for mammalian PGI. Values presented represent the average (±S.E.M.) of at least three independent experiments.

gold coated cover slips increased their phagokinetic motility by approximately twofold. Neither bacterial nor yeast PGI induced NIH-3T3 motility (Fig. 3B).

The inability of the yeast and bacterial forms of PGI to compete for binding to cell surface AMF-R and to stimulate cell motility argues that regions of the mammalian PGI structure outside of the active site are implicated in its cytokine activity [20]. Sequence comparison allowed us to identify regions in the mammalian PGI sequence that show significant differences with not only bacterial PGI but also with yeast PGI. We prepared synthetic peptides corresponding to the most distinct sequences of the mammalian protein that were localized to peripheral regions of the PGI structure [20]. These included the N-terminal region (aa 1–10), the hook region (aa 443–460), and the C-terminal region (aa 547–557) as well as the third loop region (aa 362–373) [20]. At concentrations up to 1 mg/ml, none of the selected peptides competed with the binding and endocytosis of rabbit PGI (Fig. 4).

4. Discussion

The demonstration here that neither bacterial nor yeast PGI, that both express the highly conserved PGI active site, are able to bind to the cell surface receptor for PGI nor stimulate the motility of NIH-3T3 fibroblasts argues that the active site of this glycolytic enzyme is not sufficient for receptor binding and does not exhibit a selective lectin function. Indeed, while gp78/AMF-R was originally identified for its *O*-linked glycosylation [28,29], it is difficult to imagine that sugar binding alone can be receptor-specific.

Our data conflict with prior studies showing that bacterial PGI can stimulate the motility of mouse CT-26 colon cancer cells [17,19]. The basis for the discrepancy remains unclear and could potentially be related to cell line to cell line differences. However, the inability of bacterial PGI to be endocytosed, to compete with the endocytosis of mammalian PGI or to stimulate the motility of NIH-3T3 fibroblasts demonstrates that it is not binding to the same receptor. Alternative receptors for PGI have yet to be identified although it should be noted that two affinities for PGI cell surface binding have been described and proposed to be related to the metastatic potential of the cell lines studied [30]. Certainly, recent studies describing PGI as an autoantigen on the endothelial cell surface and on sperm suggest that PGI may recognize cell surface molecules other than AMF-R [31,32]. It should also be noted that following its internalization to MVBs PGI associates with fibronectin fibrils, an association that is receptor-mediated but that may potentially implicate distinct fibronectin recognition sites [25].

The ability of inhibitors of the isomerase activity of PGI to block its cytokine function [1,7,23] in the absence of significant conformational changes in the structure of the mammalian enzyme in the presence of inhibitors [18,21,33,34] supports a role for the active site in its cytokine function. It

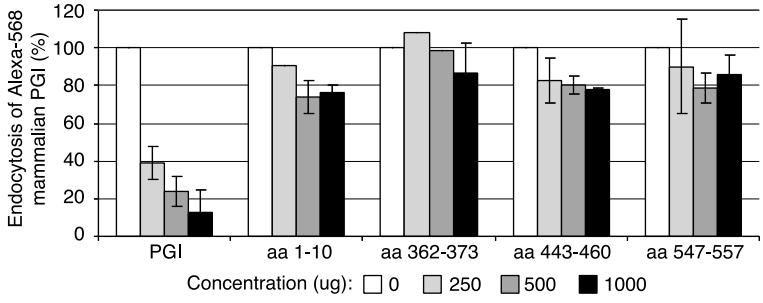

Fig. 4. Select peptides corresponding to peripheral regions of mammalian PGI do not compete for receptor binding. NIH-3T3 cells were incubated with 5 μg/ml of Alexa-568-conjugated mammalian PGI for 30 min at 37°C in the presence of 0, 250, 500 or 1000 μg/ml of either unlabeled mammalian PGI or peptides aa 1–10 (N-terminal), aa 362–373 (loop region), aa 443–460 (hook region), and aa 547–557 (C-terminal region) [20] of the mouse PGI sequence. Endocytosis of Alexa-568-conjugated mammalian PGI was quantified and is presented as the percent of endocytosis in the absence of competitor. Each value represents the average (±S.E.M.) of at least three independent experiments except for aa 1–10 and aa 362–373 at 250 μg/ml ($n=2$).

remains possible that subtle modification, below the resolution of the crystallographic methods used, of the structure of peripheral PGI regions may disrupt receptor binding. The partial inhibition by bacterial and yeast PGI of the endocytosis of mammalian PGI (Fig. 2) may reflect low affinity binding to the receptor via the conserved binding site. Furthermore, while the inability of select peptides, corresponding to distinct peripheral PGI regions [20], to inhibit PGI internalization (Fig. 4) can be considered neither complete nor conclusive, it does support the idea that receptor binding domain of PGI is a complex motif that may include elements of the active site.

PGI is an example of a 'moonlighting' protein that exhibits multiple cellular functions [35,36] and the data presented here support the evolutionary acquisition of an additional cytokine function for this glycolytic enzyme. Future studies of the receptor binding motifs of this protein should necessarily focus not only on the active site but also on PGI domains that are specific to the mammalian form of the enzyme.

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References

[1] Watanabe, H., Takehana, K., Date, M., Shinozaki, T. and Raz, A. (1996) Cancer Res. 56, 2960–2963.
[2] Chaput, M., Claes, V., Portetelle, D., Cludts, I., Cravador, A., Burny, A., Gras, H. and Tartar, A. (1988) Nature 332, 454–457.
[3] Faik, P., Walker, J.I.H., Redmill, A.A.M. and Morgan, M.J. (1988) Nature 332, 455–457.
[4] Xu, W., Seiter, K., Feldman, E., Ahmed, T. and Chiao, J.W. (1996) Blood 87, 4502–4506.
[5] Gurney, M.E., Apatoff, B.R. and Heinrich, S.P. (1986) J. Cell Biol. 102, 2264–2272.
[6] Gurney, M.E., Apatoff, B.R., Spear, G.T., Baumel, M.J., Antel, J.P., Brown Bania, M. and Reder, A.T. (1986) Science 234, 574–581.
[7] Zhi, J., Sommerfeldt, D.W., Rubin, C.T. and Hadjiargyrou, M. (2001) J. Bone Miner. Res. 16, 1994–2004.
[8] Liotta, L.A., Mandler, R., Murano, G., Katz, D.A., Gordon, R.K., Chiang, P.K. and Schiffman, E. (1986) Proc. Natl. Acad. Sci. USA 83, 3302–3306.
[9] Silletti, S., Watanabe, H., Hogan, V., Nabi, I.R. and Raz, A. (1991) Cancer Res. 51, 3301–3311.
[10] Nabi, I.R., Watanabe, H. and Raz, A. (1990) Cancer Res. 50, 409–414.
[11] Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I.R. and Raz, A. (1991) J. Biol. Chem. 266, 13442–13448.
[12] Fang, S., Ferrone, M., Yang, C., Jensen, J.P., Tiwari, S. and Weissman, A.M. (2001) Proc. Natl. Acad. Sci. USA 98, 14422–14427.
[13] Shimizu, K. et al. (1999) FEBS Lett. 456, 295–300.
[14] Silletti, S. and Raz, A. (1996) Curr. Top. Microbiol. Immunol. 213/II, 137–169.
[15] Leclerc, N., Vallée, A. and Nabi, I.R. (2000) J. Neurosci. Res. 60, 602–612.
[16] Luo, Y. et al. (2002) J. Neurochem. 80, 354–361.
[17] Chou, C.-C., Sun, Y.-J., Meng, M. and Hsiao, C.-D. (2000) J. Biol. Chem. 275, 23154–23160.
[18] Arsenieva, D., Hardre, R., Salmon, L. and Jeffery, C.J. (2002) Proc. Natl. Acad. Sci. USA 99, 5872–5877.
[19] Sun, Y.J., Chou, C.C., Chen, W.S., Wu, R.T., Meng, M. and Hsiao, C.D. (1999) Proc. Natl. Acad. Sci. USA 96, 5412–5417.
[20] Jeffery, C.J., Bahnson, B.J., Chien, W., Ringe, D. and Petsko, G.A. (2000) Biochemistry 39, 955–964.
[21] Read, J., Pearce, J., Li, X., Muirhead, H., Chirgwin, J. and Davies, C. (2001) J. Mol. Biol. 309, 447–463.
[22] Shaw, P.J. and Muirhead, H. (1977) J. Mol. Biol. 109, 475–485.
[23] Funasaka, T., Haga, A., Raz, A. and Nagase, H. (2001) Biochem. Biophys. Res. Commun. 285, 118–128.
[24] Benlimame, N., Le, P.U. and Nabi, I.R. (1998) Mol. Biol. Cell 9, 1773–1786.
[25] Le, P.U., Benlimame, N., Lagana, A., Raz, A. and Nabi, I.R. (2000) J. Cell Sci. 113, 3227–3240.
[26] Le, P.U., Guay, G., Altschuler, Y. and Nabi, I.R. (2002) J. Biol. Chem. 277, 3371–3379.
[27] Gracy, R.W. and Tilley, B.E. (1975) Methods Enzymol. 41, 392–400.
[28] Nabi, I.R. and Raz, A. (1987) Int. J. Cancer 40, 396–401.
[29] Nabi, I.R. and Raz, A. (1988) Cancer Res. 48, 1258–1264.
[30] Niinaka, Y., Haga, A., Negishi, A., Yoshimasu, H., Raz, A. and Amagasa, T. (2002) Oral Oncol. 38, 49–55.
[31] Yakirevich, E. and Naot, Y. (2000) Biol. Reprod. 62, 1016–1023.
[32] Schaller, M., Burton, D.R. and Ditzel, H.J. (2001) Nat. Immunol. 2, 746–753.
[33] Lee, J.H., Chang, K.Z., Patel, V. and Jeffery, C.J. (2001) Biochemistry 40, 7799–7805.
[34] Jeffery, C.J., Hardre, R. and Salmon, L. (2001) Biochemistry 40, 1560–1566.
[35] Jeffery, C.J. (1999) Trends Biochem. Sci. 24, 8–11.
[36] Smalheiser, N.R. (1996) Mol. Biol. Cell 7, 1003–1014.