The enzymatic activity of phosphoglucose isomerase is not required for its cytokine function

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Abstract PGI is a housekeeping gene encoding phosphoglucose isomerase (PGI) a glycolytic enzyme that also functions as a cytokine (autocrine motility factor (AMF)/neuroleukin/maturation factor) upon secretion from the cell and binding to its 78 kDa seven-transmembrane domain receptor (gp78/AMF-R). PGI contains a CXXC motif, characteristic of redox proteins and possibly evolutionarily related to the CC and CXC motif of the chemokine gene family. Using site-directed mutagenesis, single- and double-deletion (CXC, CC) mutants were created by deleting amino acids 331 and 332 of human PGI, respectively. The mutant proteins lost their enzymatic activity; however, neither of the deletions augmented the proteins' binding affinity to the receptor and all maintained cytokine function. The results demonstrate that the enzymatic activity of PGI is not essential for either receptor binding or cytokine function of human PGI.

Key words: Phosphoglucose isomerase; Autocrine motility factor

1. Introduction

Cell migration is an essential aspect of normal and pathological processes, including embryonic development, wound healing, inflammation, and tumor cell invasion [1–7]. Autocrine motility factor (AMF) is a tumor-secreted polypeptide identified by its ability to induce the migration of the AMF-producing cells [1,8]. Molecular cloning has identified AMF as phosphoglucose isomerase (PGI) [1,9], the cytokine neuroleukin [10], the maturation factor [9–13], and the serine proteinase inhibitor [14]. PGI (EC 5.3.1.9) catalyzes the interconversion of D-fructose-6-phosphate and D-glucose-6-phosphate [15]. Inhibitors of PGI isomerase activity are also able to inhibit its cytokine function [1]. Crystal structures of mammalian and bacterial PGI reveal extensive conservation of the active site but significant differences in other regions of the protein [16–18]. Reports that bacterial PGI stimulates the motility of murine cancer cells further implicated the sugar-binding active site in receptor recognition [17–19]. However, this was challenged based on the demonstration that the cytokine activity of PGI is specific only for the mammalian form of the enzyme, implying that the active site per se is not sufficient for the cytokine activity of the protein [20].

The receptor for AMF/PGI (gp78/AMF-R) is a seven-transmembrane domain G-protein coupled receptor [2,21]. AMF/PGI therefore resembles members of the CC, CXC gene family of chemoattractants, including macrophage inflammatory protein-1a (MIF)/RANTES and monocyte chemoattractant protein-1 (MCP-1), that signal via the CXCR family of seven-transmembrane domain G-protein coupled receptors [22,23]. PGI contains a CXXC [Cys_{330}-Phe-Glu-Cys_{333}] motif also found in the disulfide isomerase protein family [24] and MIF [25,26]. Since the integrity of a disulfide linkage is essential for MIF activity [27], and reducing agents abolish PGI function [8], we questioned whether this motif regulates either the enzymatic or cytokine functions of PGI. Single- and double-deletion mutants were created by site-directed mutagenesis and their biochemical and cellular functions compared to wild-type (wt) protein. The deletion of either or both amino acids at the X positions in the CXXC motif affected neither PGI cytokine activity nor its ability to bind to AMF-R but did result in loss of isomerase activity. Thus, the cytokine activity of PGI is independent of the active site of this multifunctional enzyme.

2. Material and methods

2.1. Cell culture and reagents

Human fibrosarcoma HT1080 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultivated as previously described [8]. Mono-specific rabbit polyclonal antibodies (anti-AMF) were generated as previously described [11]. Fructose-6-phosphate, NADP, glucose 6-phosphate dehydrogenase, erythrose 4-phosphate (E4P) was purchased from Sigma (St. Louis, MO, USA).

2.2. SDS–PAGE

The molecular weight of AMF and its mutants was determined by running 2 μg of protein on 8% SDS–polyacrylamide gels alongside prestained molecular weight markers (Gibco-BRL, Bethesda, MD, USA).

2.3. Co-immunoprecipitation of PGI/AMF and AMF-R

In order to examine whether wt, CC, and CXC bind to the same receptor, HT1080 cell lysates were immunoprecipitated with anti-

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Abbreviations: AMF, autocrine motility factor; E4P, erythrose 4-phosphate; PGI, phosphoglucose isomerase

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AMF-R in IP buffer (final concentration 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5% NP-40). Samples were incubated at 4°C for 2 h. After washing, wt, CC, CXC, or bovine serum albumin (BSA) (control) was added to each sample and incubated for 2 h at 4°C with constant rotation. Then 50 μl of protein A Sepharose beads was added to each sample for 1 h at 4°C. Samples were centrifuged, washed and separated by SDS–PAGE.

2.4. PGI activity

PGI enzymatic activity of purified recombinant human AMF was measured as previously described [28].

2.5. Cell motility assays

Random motility was assayed by the phagokinetic method [30]. Directional cell motility assay was measured using a 48-well Boyden chamber as described in [7].

2.6. Scatchard analysis

Binding of ^{125}I-labeled PGI (iodogen method) to HT1080 cells was determined and receptor affinity (K_d value) and receptor concentration (R value) were determined using Scatchard's method [29].

2.7. Homology modeling and graphic program

The program MODELLER [31] was used for homology modeling calculations using the crystal structure of inhibitor-free PGI [18] as a template. Ribbon diagrams were produced with the program ViewerProTM (Accelrys, San Diego, CA, USA).

3. Results

We and others have reported that PGI is a multifunctional protein [1,9–15]. Here, we questioned the dependence of PGI cytokine function on its enzymatic activity. Wild-type (wt) PGI, and single-deletion (CXC) and double-deletion (CC) mutants were constructed, expressed, and purified as previously described [32]. As seen in Fig. 1 (panel I), wt, CXC and CC PGI co-migrate in SDS–PAGE as a single protein band. Enzymatic analysis revealed that while wt PGI exhibited isomerase activity, both mutants were enzymatically inactive (Fig. 1, panel II). Next, we questioned whether enzymatic activity is associated with cytokine activity. Using two independent assays to study both random (Fig. 1, panel IIIA) and directional (Fig. 1, panel IIIB) motility, we found that wt PGI (CXXC) as well as the CXC and CC mutants at 30 ng/ml all stimulated the motility of HT1080 cells to the same levels relative to unstimulated control cells (Fig. 1, panels IIIA and IIIB). Interestingly, E4P, a specific PGI enzymatic inhibitor, affected

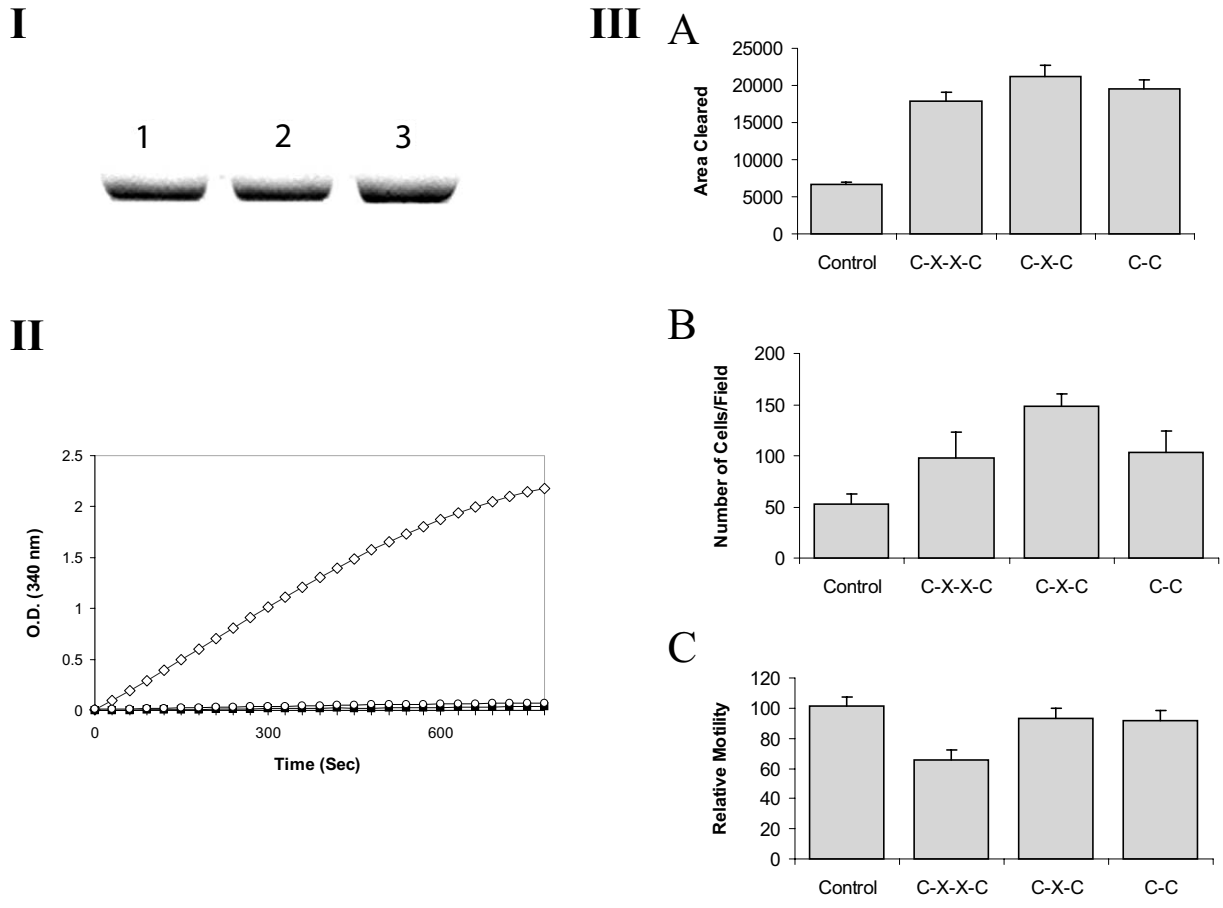

Fig. 1. I: Molecular weight determination using SDS–PAGE analysis. Recombinant human PGI protein and its mutant proteins were separated on 8% SDS gel under reducing conditions. Lane 1, wt; lane 2, CC; and lane 3, CXC. II: Spectrophotometric determination of PGI activity of wt PGI, CC, and CXC. PGI activity of wt, CC, and CXC was measured at 340 nm on the basis of reduction of NADP to NADPH. The graph is representative of three independent experiments with similar results. Open square, wt; open circle, CXC; closed square, CC. III: Effect of CXXC (wt), or CC, CXC proteins on the motility of HT1080 cells. A: Phagokinetic analysis. The area cleared by at least 20 cells was determined. The graph is representative of three independent experiments with similar results using (panel B) Boyden Chamber assay. C: Effect of E4P on the motility induced by CXXC (wt), CC or CXC proteins. The results are expressed as mean ± S.D. Random 10 fields were counted. The results are the mean ± S.D. representative of three independent experiments.

Fig. 2. Identification of wt, CC, and CXC binding receptor. HT1080 cell lysates expressing cell surface AMF-R were incubated with anti-AMF-R specific antibody and the resulting complex was incubated with protein A Sepharose beads, centrifuged and washed, then incubated with wt, CC, CXC AMF/PGI or BSA (control). The complexes were analyzed by SDS–PAGE for the presence of AMF/PGI. Lane 1, wt; lane 2, CC; lane 3, CXC; lane 4, BSA.

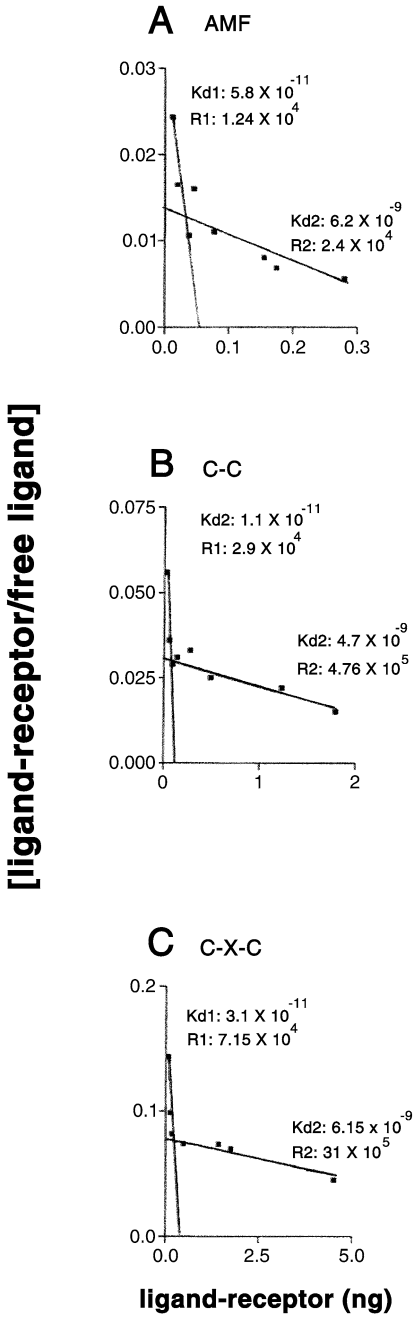

Fig. 3. Scatchard plot analysis of binding of ^{125}I-labeled wt, CC or CXC proteins to cell surface AMF-R of HT1080 cells.

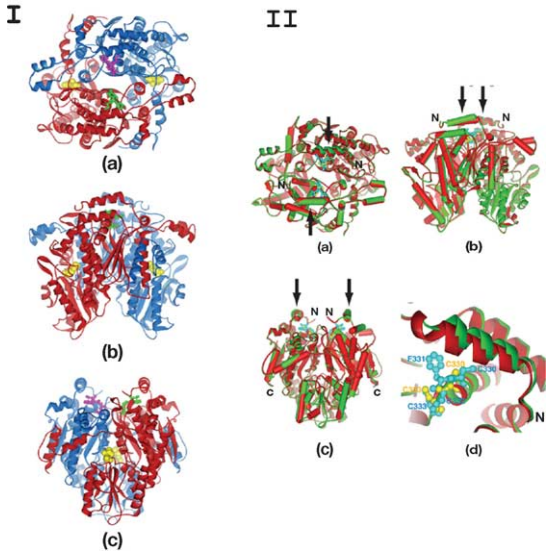

Fig. 4. I: Ribbon models showing the location of the CXXC motif on the crystal structure of wt human PGI dimer. Bound inhibitor molecules showing the location of the PGI active site are shown as yellow. The CXXC motifs are shown as ball-and-stick models and colored green and magenta for 'red' and 'blue' subunits, respectively. Ia: Top view of the dimer. Ib: Front view. Ic: Side view. II: Comparison of crystal structure of inhibitor-free wt human PGI (green) and that of the theoretical model of inhibitor-free double-deletion (del. F331G332) mutant of human PGI (red). One subunit is shown as a ribbon and the other subunit is shown as cylinders (α-helices) and arrows (β-strands). The N-terminal helices for which a significant structural movement is observed are indicated by arrows (black). The CXXC motif of wt human PGI and the CC motif of double-deletion mutant of the protein are shown as ball–stick models and colored cyan and yellow, respectively. IIa: Top view of the dimer. IIb: Front view. IIc: Side view. IId: Close view of the mutation site. Note that a significant movement of the N-terminal helix is observed.

the induction of cell migration of only wt PGI protein with no effect on either the basal or CC or CXC induced cell migration (Fig. 1, panel III 3C).

To determine whether the PGI mutants' cytokine activity relates to receptor binding, the proteins were incubated with gp78/AMF-R derived from a lysate of HT1080 cells. AMF-R was immunoprecipitated with specific antibodies and the precipitate probed by Western analysis for both PGI and AMF-R. As seen in Fig. 2, all PGI forms interact with immunoprecipitated AMF-R. Next, to establish ligand–receptor binding constants, Scatchard analysis was performed to determine the affinity (K_d) and the binding maximum (B_{max}) for PGI to its receptor (Fig. 3). The high affinity binding of wt PGI to its receptor on HT1080 cells was similar to that observed for the deletion mutants; however, B_{max} was higher for the mutants than for wt PGI (CXC > CC > CXXC).

The effect of amino acid deletions in the CXXC motif on three-dimensional models of PGI structure was determined. Human PGI is a dimer [18] presented as ribbon diagrams with the two monomers in red and blue and is spherical in shape (Fig. 4, panel I). Bound inhibitor molecules are shown in yellow to localize the PGI active site. The CXXC motifs are shown as ball-and-stick models in green and magenta for the red and blue subunits, respectively, and are located outside of the active site of the enzyme. Fig. 4 (panel II) presents a comparison of the crystal structure of inhibitor-free wt human

PGI/AMF (red) and a theoretical model of the inhibitor-free CC mutant (green). One subunit is shown as a ribbon and the other subunit is shown as cylinders (α-helices) and arrows (β-strands). The CXXC motif of wt human PGI/AMF and the CC motif of double-deletion mutant of human AMF are shown as ball–stick models and colored cyan and yellow, respectively, and significant structural movement of the N-terminal helices is predicted (black arrows).

4. Discussion

AMF is a tumor-secreted cytokine that regulates cellular growth and motility by a receptor-mediated pathway [33] that is implicated in the most devastating aspect of cancer: metastasis [34]. The identity of AMF with the ubiquitous cytosolic enzyme PGI is supported by reports that PGI is also a neurokine and lymphokine [10,12,35]. However, while the motifs in the PGI sequence implicated in its enzymatic activity are well characterized [36] the structural motifs that regulate its cytokine activity and receptor binding remained to be determined. The human PGI sequence contains a unique CXXC motif between amino acids 330 and 333, resembling, in part, the CXC and CC motifs of MIF and MCP-1 [23,37]. However, deletion mutants of the CXXC intra-domain of this motif did not result in loss of cytokine activity or binding to gp78/AMF-R, but did result in a complete loss of the isomerase activity.

PGI is nearly ubiquitous in evolution during which a cluster of about 40 residues has remained conserved, while most residues outside the active site were altered [31]. It was previously reported that bacterial PGI exhibited cytokine ability, which, together with the ability of inhibitors of PGI isomerase function to block cytokine activity, led to the conclusion that the substrate and receptor binding sites of PGI are similar or located within close proximity of each other [17,19]. However, recently, it was reported that the cytokine function of the enzyme has evolved during evolution independently of the enzymatic activity and that the conserved sugar-binding domain is not sufficient for its cytokine activity [20]. Loss of enzymatic but not cytokine activity of the CXC and CC mutants confirms this result. Furthermore, the failure of the sugar inhibitor to block cytokine activity of the CXC and CC protein mutants provides the first direct evidence that the sugar recognition site of PGI is not critical for ligand–receptor recognition and that the enzymatic active site is not directly involved in cytokine activity.

Specific binding of 5-phospho-arabinose involves a network of polar interactions [16,17,19]. The mammalian-specific CXXC motif and associated disulfide bridges are shown here to be critical for the integrity of the active site of mammalian PGI and this motif could therefore be a mammalian-specific regulator of PGI isomerase activity. Interestingly, mutations associated with hemolytic anemia are found near the CXXC motif, specifically Leu338, Arg346, Gly323, and Gln342, and are predicted to disrupt the structure of the PGI dimer [38]. The proximity of the CXXC motif to the N-terminal region of PGI is predicted here to induce structural movement of the N-terminal helices that will affect the overall conformation of the protein, thereby affecting its isomerase activity.

Of note, the overall level of sequence homology between bacterial and mammalian PGI in the N-terminal region is quite low and striking structural differences are found in this region [19]. The N-terminal region as well as the evolutionarily distinct C-terminal domain and peripheral hook and loop regions are candidates for receptor-interacting domains of mammalian PGI exhibiting cytokine activity [20]. Additional deletions, mutations or amino acid substitutions in these regions will be required to directly determine the exact structural motif controlling receptor binding and cytokine activity of PGI. To summarize, we provide the first experimental evidence of dichotomy between the enzymatic and cytokine activities of PGI. The fact that local conformation changes due to condensation of the distance between the cysteine residues of the CXXC motif of PGI disrupt enzymatic activity but not receptor interaction shows definitively that the enzymatic activity of PGI is not involved in its cytokine function.

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References

[1] Watanabe, H., Takehana, K., Date, M., Shinozaki, T. and Raz, A. (1996) Cancer Res. 56, 2960–2963.
[2] Nabi, I.R., Watanabe, H. and Raz, A. (1990) Cancer Res. 50, 409–414.
[3] Liang, M.N. and Garrison, J.C. (1991) J. Biol. Chem. 266, 13342–13349.
[4] Thomas, H. and Balkwill, F.R. (1991) Pharmacol. Ther. 52, 307–330.
[5] Boguski, M.S. and McCormick, F. (1993) Nature 366, 643–654.
[6] Genda, T., Sakamoto, M., Ichida, T., Asakura, H., Kojiro, M., Narumiya, S. and Hirohashi, S. (1999) Hepatology 30, 1027–1036.
[7] Liotta, L.A., Mandler, R., Murano, G., Katz, D.A., Gordon, R.K., Chiang, P.K. and Schiffmann, E. (1986) Proc. Natl. Acad. Sci. USA 83, 3302–3306.
[8] Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I.R. and Raz, A. (1991) J. Biol. Chem. 266, 13442–13448.
[9] Chaput, M., Claes, V., Portetelle, D., Cludts, I., Cravador, A., Burny, A., Gras, H. and Tartar, H. (1988) Nature 332, 454–457.
[10] Gurney, M.E., Heinrich, S.P., Lee, M.R. and Yin, S.H. (1986) Science 234, 566–574.
[11] Niinaka, Y., Paku, S., Haga, H., Watanabe, H. and Raz, A. (1998) Cancer Res. 58, 2667–2674.
[12] Xu, W., Seither, K., Feldman, E., Ahmed, T. and Chiao, J.W. (1996) Blood 87, 4502–4506.
[13] Chiao, J.W., Xu, W., Seither, K., Feldman, E. and Ahmed, T. (1999) Leuk. Res. 23, 13–18.
[14] Cao, M.J., Osatomi, K., Matsuda, R., Ohkuba, M., Hara, K. and Ishihara, T. (2000) Biochem. Biophys. Res. Commun. 272, 485–490.
[15] Harrison, R.A.P. (1974) Anal. Biochem. 61, 500–507.
[16] Arsenieva, D., Hardre, R., Salmon, L. and Jeffery, C.J. (2002) Proc. Natl. Acad. Sci. USA 99, 5872–5877.
[17] Chou, C.C., Sun, Y.J., Meng, M. and Hsiao, C.D. (2000) J. Biol. Chem. 275, 23154–23160.
[18] Tanaka, N., Haga, A., Uemura, H., Akiyama, H., Funasaka, T., Nagase, H., Raz, A. and Nakamura, K.T. (2002) J. Mol. Biol. 318, 985–997.
[19] Sun, Y.J., Chou, C.C., Chen, W.S., Wu, R.T. and Meng, M. (1999) Proc. Natl. Acad. Sci. USA 96, 5412–5417.
[20] Amraei, M. and Nabi, I.R. (2002) FEBS Lett. 525, 151–155.
[21] Shimizu, K., Tani, M., Watanabe, H., Nagamachi, Y., Niinaka, Y., Shiroishi, T., Ohwada, S., Raz, A. and Yokota, J. (1999) FEBS Lett. 456, 295–300.
[22] Boulay, F., Naik, N., Giannini, E., Tardif, M. and Brouchon, L. (1997) Ann. N.Y. Acad. Sci. 832, 69–84.
[23] Gutkind, J.S. (1998) J. Biol. Chem. 273, 1839–1842.

[24] Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. and Creighton, T.E. (1997) Curr. Biol. 7, 239–245.
[25] Kleemann, R., Kapurniotu, A., Frank, R.W., Gessner, A., Mischke, R., Flieger, O., Juttner, S., Brunner, H. and Bernhagen, J. (1998) J. Mol. Biol. 280, 85–102.
[26] Swope, M., Sun, H.W., Blake, P.R. and Lolis, E. (1998) EMBO J. 17, 3534–3541.
[27] Kleeman, nR., Kapurniotu, A., Frank, R.W., Gessner, A., Mischke, R., Flieger, O., Juttner, S., Brunner, H. and Bernhagen, J. (1998) J. Mol. Biol. 280, 85–102.
[28] Gracy, R.W. and Tilley, B.E. (1975) Methods Enzymol. 41, 392–400.
[29] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.
[30] Silletti, S., Watanabe, H., Hogan, V., Nabi, I.R. and Raz, A. (1991) Cancer Res. 51, 3301–3311.
[31] Sali, A. and Blendell, T.L. (1993) J. Mol. Biol. 234, 779–815.
[32] Haga, A., Niinaka, Y. and Raz, A. (2000) Biochim. Biophys. Acta 1480, 235–244.
[33] Silletti, S., Paku, S. and Raz, A. (1998) Int. J. Cancer 76, 129–135.
[34] Roy, V. and Mareel, M. (1992) Trends Cell Biol. 2, 167–169.
[35] Gurney, M.E., Apatoff, B.R. and Heinrich, S.P. (1986) J. Cell Biol. 102, 2264–2272.
[36] Jeffery, C.J., Bahnson, B.J., Chien, W., Ringe, D. and Petsko, G.A. (2000) Biochemistry 39, 955–964.
[37] Arai, H. and Charo, I. (1996) J. Biol. Chem. 271, 21814–21819.
[38] Read, J., Pearce, J., Li, X., Muirhead, H., Chirgwin, J. and Davies, C. (2001) J. Mol. Biol 309, 447–463.