Properties of an insulin receptor with an IGF-1 receptor loop exchange in the cysteine-rich region

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Abstract The insulin receptor (IR) and the insulin-like growth factor-I receptor (IGF-1R) show differential binding of insulin and IGFs. The specificity determinants for IGF-1 binding are known to be located in the cysteine-rich (Cys-rich) region between residues 223 and 274 of human IGF-1R, which includes a loop that protrudes into the putative ligand binding site. In this report we have replaced residues 260–277 of human IR with residues 253–266 of the human IGF-1R to produce an IR-based, cysteine loop exchange chimaera, termed hIR-Cys loop exchange (CLX), in which all 14 amino acid residues in the exchanged loop differ from wild-type insulin receptor. This loop exchange had a detrimental effect on the efficiency of pro-receptor processing and on the binding of the mouse monoclonal antibody 83-7. However, this antibody, which binds hIR but not hIGF-1R, was still capable of immunoprecipitating the mature chimaeric receptor, indicating that the conformational epitope recognised by this antibody is not primarily determined by the loop region exchanged. The loop exchange did not significantly affect the ability of insulin to displace bound radiolabelled insulin, but increased the capacity of IGF-1 to competitively displace labelled insulin by at least 10 fold.

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Key words: Insulin receptor; IGF-1; Cysteine; Loop exchange

1. Introduction

The ectodomains of members of the insulin receptor (IR) family are dimers made up of several modular units. The N-terminal half of the ectodomain contains two homologous domains, L1 and L2, separated by a Cys-rich region (Cys159–Cys308) [1–3]. The C-terminal half consists of three fibronectin type III domains, the second of which contains a large insert domain that includes the protease cleavage site that generates the α and β chains [4–7].

The role of different extracellular regions of these receptors in ligand binding has been established through studies involving either chemical cross-linking, receptor fragments or chimaeric receptors. Chemical cross-linking with derivatised ligands labels only the α-chains of the receptor [8–10]. Furthermore, expressed α-chains [11], or α-chain mini receptors consisting of the L1/Cys-rich/L2 domains fused to the last 16 residues of the α-chain [12,13], bind ligand with comparable affinities to ectodomain dimers. The data obtained from IR/IGF-1R chimaeras of both full length receptor and ectodomain established the importance of the Cys-rich region in controlling IGF-1 binding specificity, but not insulin binding. These studies mapped the determinants of IGF-1 specificity to residues 223–274 in the Cys-rich region of IGF-1R [14–18]. These residues correspond to modules 4, 5 and 6 in the Cys-rich region of IGF-1R with modules 5 and 6 located on one side of the central cavity and possibly forming part of the ligand binding site [3]. Module 6 has a mobile loop (residues 255–265) which extends into the central cavity of the L1/Cys-rich/L2 fragment and is the most divergent Cys-rich module in terms of sequence identity compared to IR, with 25 of the 31 residues being different. The corresponding region in IR is larger, containing four additional residues and a disulphide bond not present in IGF-1R [2] and has a markedly different net charge with five basic residues and one acidic residue compared to six acidic residues and one basic residue in module 6 of hIGF-1R [3]. Thus the larger size and positive charge of the loop in hIR may serve to perturb binding of IGF-1, so reducing the affinity of the receptor for this ligand, while maintaining tight binding of the smaller insulin molecule.

To test this hypothesis, a mutant hIR was constructed in which residues 260–277 of hIR were replaced by residues 253–266 of IGF-1R (Fig. 1) and the effects on ligand binding to this chimaeric receptor, termed hIR-Cys loop exchange (CLX), were examined. In addition, the epitope for Mab 83-7 [19] which is known to be located within residues 191–297 of the Cys-rich region of IR [11,20], has been further mapped using this chimaera. Four of the seven sequence differences between the mouse and human IR sequences within the region 191–297 [21–23] are located within the variable loop of module 6 that has been exchanged in this hIR-CLX chimaera, making it a useful construct to further define the 83-7 epitope.

2. Materials and methods

2.1. Mutagenesis

The DNA used in these studies was derived from a full-length hIR cDNA from plasmid peT [24]. A 1.0 kb *Eco*RI fragment of hIR cDNA from plasmid peT, encoding the N-terminal sequence to amino acid residue 296 [21], was introduced into M13mp18 [25] for mutagenesis. Oligonucleotide-directed in vitro mutagenesis using the USB-T7 Gen™ in vitro mutagenesis kit was employed to exchange nucleotide sequence encoding amino acid residues 260–277 inclusive of hIR with the sequence encoding residues 253–266 inclusive of IGF-1R (Fig. 1). Mutant clones were identified by colony hybridisation [26] using an oligonucleotide probe 5′-end labelled with [γ-^{32}P]ATP, and were confirmed by sequence determination [27].

A vehicle for mammalian cell expression of hIR cDNA with which the mutated fragment could be exchanged was prepared from expres-

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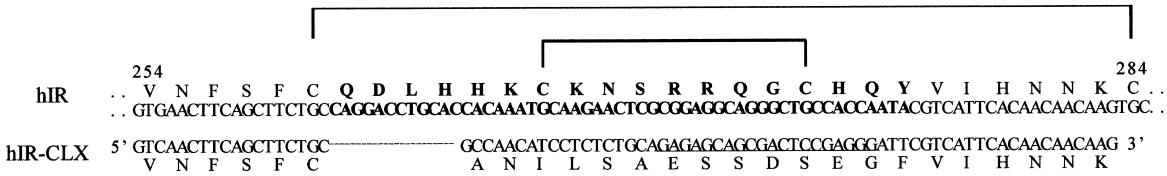

Fig. 1. Mutagenesis of hIR cDNA. Residues 254–284 of the amino acid sequence of hIR are shown, and immediately below is the nucleotide sequence of hIR cDNA encoding this region; the nucleotide sequence changed in the mutant through site-directed mutagenesis is in bold. The 78 residue oligonucleotide used in mutagenesis and the amino acid sequence of the resulting hIR/IGF-1R chimaera obtained from the construction are shown below the hIR cDNA sequence. The segment of this mutant oligonucleotide sequence used as a probe, to identify clones that had incorporated the mutation, is underlined.

sion vector pEE14 (Celltech, Slough, UK [28] modified by deletion of the *Eco*RI restriction site through fill-in and religation. The hIR cDNA for this construct was from plasmid peT, and was inserted into the polylinker of the modified pEE14 expression vector as a *Hin*dIII–*Xba*I fragment. The mutagenised DNA cloned in M13mp18 was exchanged with the corresponding part of hIR cDNA of this expression vehicle by exchange of the 1.0 kb *Eco*RI fragments. These procedures produced an expression vehicle encoding a mutant hIR designated hIR-CLX, in which a segment of the sequence which forms a loop within module 6 of the Cys-rich domain was replaced by the shorter loop of the IGF-1R sequence with four fewer residues and lacking the nested disulphide bond of the hIR sequence.

2.2. Cell culture, DNA transfection and protein analysis

For transient transfection assays, human 293T and African green monkey-derived COS fibroblasts maintained in DMEM plus 10% foetal calf serum were transfected with plasmid DNA using FuGENE (Roche Molecular Biochemicals) according to the manufacturers instructions. For ligand-binding studies, cells were seeded at 10^6 cells per 10 cm plate prior to transfection. 24 h after transfection, cells were harvested from 10 cm plates by trypsination and replated into poly-D-lysine treated 96 well plates (Becton Dickinson) at 5×10^4 cells per well (293T cells) or standard 24 well plates (COS cells). These cells were cultured for a further 24 h prior to serum starvation for 4–5 h and ligand binding studies (see below). For immunoprecipitation studies, 293T cell cultures were seeded at 3×10^5 cells per 3.5 cm plate. 48 h after transfection, cells were solubilised in NP-40 lysis buffer [29]. Solubilised receptors were immunoprecipitated from cell lysates with the conformation-dependent, anti-α chain-specific hIR monoclonal antibodies 83-7 or 83-14 [19]. Immune complexes were collected on protein A-Sepharose beads (Zymed Laboratories), fractionated by SDS–polyacrylamide gel (7.5%) under reducing conditions and the proteins transferred to nitrocellulose membranes [29]. Immunoblotting of membranes was performed in 3% skim milk in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) using the hIR β-chain-specific monoclonal antibody, CT-1 [30]. Bound antibody was detected by probing with horseradish peroxidase-conjugated, rabbit anti-mouse IgG (Santa Cruz Biotechnology) followed by detection with Super Signal West Pico chemiluminescent substrate (Pierce).

2.3. Ligand binding assays

For binding analysis, adherent transiently transfected cells were incubated in a total volume of 100 μl (96 well plates; 293T cells) or 300 μl (24 well plates; COS cells) with 40 pM [^{125}I-Tyr A14]insulin (Amersham) and varying concentrations of unlabelled ligand in binding buffer (100 mM HEPES, 120 mM NaCl, 15 mM Na acetate, 1.2 mM $MgSO_4$, 10 mM glucose, 0.5% w/v BSA, pH 7.8) for 1.5 h at 15°C. Subsequently the cellular supernatants were removed by aspiration and the cells were washed with ice-cold PBS prior to lysis with buffer containing 20 mM HEPES, 150 mM NaCl, 10% v/v glycerol, 1% w/v Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, pH 7.5. Bound counts were determined in a Packard γ-counter after transfer of the cell lysates to individual tubes. Assays were performed three times, with each data point being determined in triplicate and K_d and IC_{50} values ± standard error of the mean are reported. Binding constants were determined using the Ligand program [31].

3. Results

The effects of replacing residues 260–277 of human insulin receptor (hIR) with residues 253–266 of hIGF-IR (see Fig. 1) on proreceptor processing and assembly in 293T cells were analysed by immunoprecipitation and immunoblotting. Immunoprecipitation of wild-type hIR from transfected cell lysates with either Mab 83-14 or Mab 83-7, followed by immunoblotting with the β-chain Mab CT-1, showed only processed β-chain subunits (Fig. 2, lanes 2 and 5), although proreceptor is discernible upon longer exposure (data not shown). Similarly, direct blotting of lysates expressing wild-type receptor with Mab CT-1 indicated efficient proreceptor processing (Fig. 2, lane7). In contrast, the chimaeric proreceptor (hIR-CLX) is processed inefficiently, as indicated by the disproportionate amount of proreceptor relative to β-chain detected by Mab CT-1 in cell lysates of transfected cells (Fig. 2, lane 8), and following immunoprecipitation with Mab 83-14 (Fig. 2, lane 3). Mab 83-7 still binds hIR-CLX but exhibits a reduced affinity for the chimaeric receptor compared to the wild-type receptor, based on immunoprecipitation (Fig. 2, lane 5 vs 6) and fails to immunoprecipitate the hIR-CLX proreceptor, in contrast to 83-14 (Fig. 2, lane 3 vs 6).

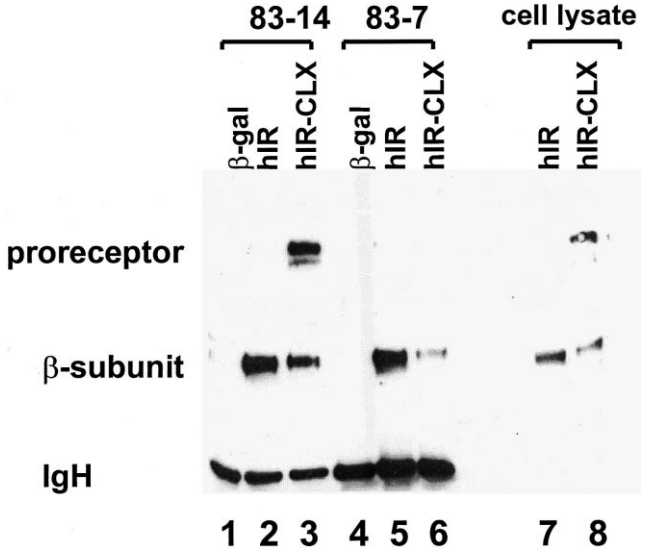

Fig. 2. Immunoreactivity of wild-type and chimaeric insulin receptors with Mabs 83-7 and 83-14. 293T fibroblasts transfected with plasmid DNA encoding wild-type (hIR), chimaeric (hIR-CLX) receptors and a negative control vector (β-gal) were lysed and immunoprecipitated with monoclonal antibodies (83-14 or 83-7) specific for the receptor α-subunit. Immunoprecipitated samples (lanes 1–6), together with aliquots of total cell lysate from receptor-transfected cells (lanes 7,8), were immunoblotted with the β-chain-specific monoclonal antibody, CT-1. IgH refers to the IgG heavy chain of the precipitating Mab.

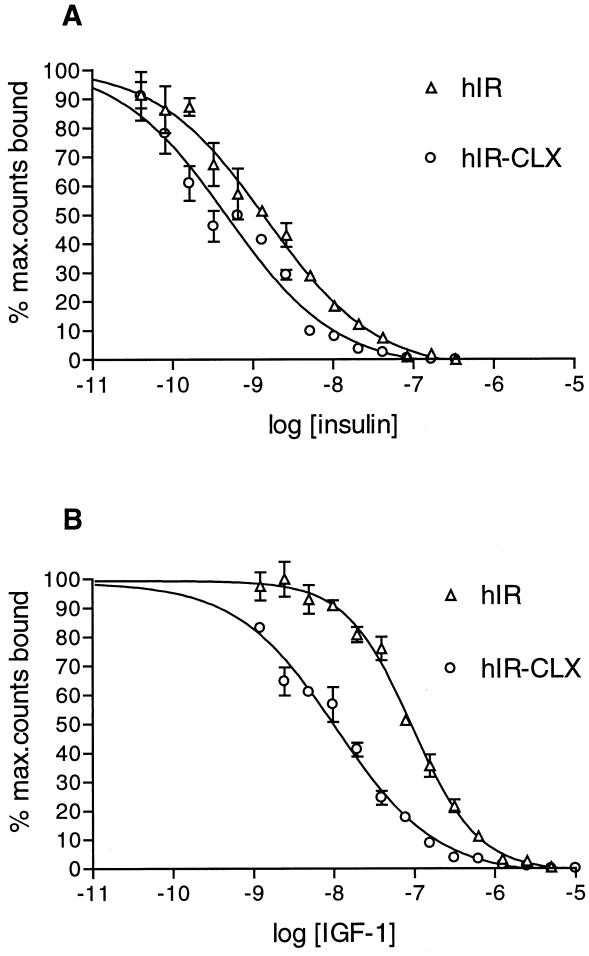

Fig. 3. Competition binding curves for ^{125}I-insulin binding to 293 cells expressing hIR and hIR-CLX. 293T fibroblasts expressing hIR or hIR-CLX were incubated with ^{125}I-insulin and varying concentrations of unlabelled insulin or IGF-1. The amount of ^{125}I-insulin bound, as a percentage of ^{125}I-insulin bound in the absence of unlabelled hormone, is plotted against the concentration of unlabelled insulin (A) or IGF-1 (B). The plots shown are from a representative experiment. Error bars indicate the standard error of the mean. Curve fitting to data was performed using non-linear regression analysis on GraphPad Prism (GraphPad Software).

Competition binding assays, in which ^{125}I-insulin is displaced by unlabelled insulin, showed that insulin binding by hIR-CLX and hIR did not differ significantly (Fig. 3A). Analysis of the binding data produces similar dissociation constants for both receptors (hIR: $K_{d1} = 2.1 \pm 0.9 \times 10^{-10}$, $K_{d2} = 7.2 \pm 2.0\ 10^{-9}$ and hIR-CLX: $K_{d1} = 1.1 \pm 0.1 \times 10^{-10}$, $K_{d2} = 1.6 \pm 0.6\ 10^{-9}$). However, competitive binding assays using ^{125}I-insulin and unlabelled IGF-1 as the competing ligand show a substantial (10 fold) difference between hIR and hIR-CLX (Fig. 3B); IGF-1 displacing ^{125}I-insulin bound to hIR-CLX more easily than ^{125}I-insulin bound to hIR (hIR-CLX, $IC_{50} = 5.7 \pm 2.0 \times 10^{-9}$ M; hIR, $IC_{50} = 5.7 \pm 2.2 \times 10^{-8}$ M). Competitive binding studies were also carried out in triplicate on hIR and hIR_CLX expressed in COS cells. There was no difference in capacity of insulin to displace ^{125}I-insulin from either construct (not shown) while the capacity of IGF-1 to displace ^{125}I-insulin from hIR_CLX was 12 times greater than its ability to displace radiolabelled ligand from wild-type hIR (not shown).

4. Discussion

The Cys-rich region of IGF-1R has been shown to be an important determinant of IGF-1 binding specificity. An analysis of 12 IR/IGF-1R chimaeras showed that the IGF-1 specificity determinants were located between IGF-1R residues 132–315 [17,18]. IR-based chimaeras, containing residues 1–514, 1–315 or 132–315 from IGF-1R, bound IGF-1 with high affinity while chimaeras 132–315 from IGF-1R did not [17,18]. The binding specificity determinant could be further narrowed to residues 184–279, based on the binding characteristics of a series of IR/IGF-1R ectodomain chimaeras: a chimaera containing residues 1–284 from IGF-1R, bound IGF-1 with high affinity [15], another chimaera containing IGF-1R residues 1–180 bound neither ligand [16], while a chimaera containing residues 184–279 of IGF-1R bound both insulin and IGF-1 with high affinity [16]. The differential binding specificities of IR-based chimaeric whole receptors containing residues 1–217 of IGF-1R (which bound insulin preferentially) and 1–274 of IGF-1R (which bound IGF-1 preferentially), mapped the location of the IGF-1 specificity determinants to residues 223–274 of IGF-1R, since residues 218–222 are conserved between IGF-1R and IR [14].

In this report we have further narrowed the region controlling IGF-1 discrimination to the variable loop (IGF-1R residues 253–266) in module 6 of the Cys-rich region. The chimaeric receptor hIR-CLX was not significantly different from wild-type hIR in terms of insulin binding affinity, but was more amenable to displacement of ^{125}I-insulin by IGF-1 than was hIR. High-resolution structural information is required to establish whether this loop makes direct contact with IGF-1 in the ligand/receptor complex or whether the poorer binding of IGF-1 by IR is due to steric constraints imposed by the loop.

Studies on IGF-1 have shown that replacement of the C region by a four residue glycine linker reduced affinity for IGF-1R by 40–100 fold [32,33] with a 10 fold reduction [32] or a small increase [33] in binding to IR. Alanine mutants of IGF-1 have implicated Arg-21, Arg-36 and Arg-37 in IGF-1R binding [34]. The region of the receptor responsible for recognising Arg-36 and Arg-37 is the Cys-rich region 217–284 [35] which has eight acidic residues not found in the corresponding positions in IR. Six of these line the putative binding pocket, including the three in the loop region which when exchanged (hIR-CLX) dramatically affected the displacement of bound insulin by IGF-1. Thus it seems likely that electrostatic interactions play an important part in IGF-1 binding, with the positively charged C region having an orientational role in the binding of IGF-1 to the acidic patch of residues in the central region of the Cys-rich domain.

The hIR-CLX chimaera provided an opportunity to further map the residues involved in the epitope for Mab 83-7 [19]. This antibody reduced insulin binding to adipocytes by only 10% and was insulin mimetic, but at 20 times higher molar ratio than insulin [36]. The epitope of mouse Mab 83-7 resides between residues 191 and 297 in the Cys-rich region of hIR [11,20] which has seven residues different from mouse IR [23], four of which occur in the exchanged loop. The fact that hIR-CLX (in which all 14 amino acid residues encompassing the exchanged loop differ from those in the wild-type hIR), can still be recognised by Mab 83-7, indicates that the module 6 loop of hIR does not contribute substantially to the epitope.

Of the remaining three sequence differences between mouse and human IR in the region 191–297, residue 218 is adjacent to an N-linked glycosylation site at 215 and thus likely to be obscured by oligosaccharide. This leaves residues 210 in the third Cys-rich module and 236 in the fourth Cys-rich module, as the major contributors to the epitope on hIR for the mouse monoclonal antibody 83-7. In the three-dimensional structure of hIGF-1R, the residues equivalent to 210 and 236 of hIR are in close proximity at 12 Å [3], in a location that is consistent with the single molecule images of hIR/83-7 Fab complexes [37]. In addition, the closest residues of the loop are approximately 40 Å distant on an opposite face of the molecule to residues 210 and 236, and are unlikely to form part of the same epitope as residues 210 and 236.

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References

[1] Bajaj, M., Waterfield, M.D., Schlessinger, J., Taylor, W.R. and Blundell, T. (1987) Biochim. Biophys. Acta 916, 220–226.
[2] Ward, C.W., Hoyne, P.A. and Flegg, R.H. (1995) Proteins Struct. Funct. Genet. 22, 141–153.
[3] Garrett, T.P.J., McKern, N.M., Lou, M., Frenkel, M.J., Bentley, J.D., Lovrecz, G.O., Elleman, T.C., Cosgrove, L.J. and Ward, C.W. (1998) Nature 394, 395–399.
[4] O'Bryan, J.P., Frye, R.A., Cogswell, P.C., Neubauer, Z., Kitch, B., Prokop, C., Espinosa III, R., Le Beau, M.M., Earp, H.S. and Liu, E.T. (1991) Mol. Cell Biol. 11, 5016–5031.
[5] Mulhern, T.D., Booker, G.W. and Cosgrove, L. (1998) Trends Biochem. Sci. 23, 465–466.
[6] Marino-Buslje, C., Mizuguchi, K., Siddle, K. and Blundell, T.L. (1998) FEBS Lett. 441, 331–336.
[7] Ward, C.W. (1999) Growth Factors 16, 315–322.
[8] Wedekind, F., Baer-Pontzen, K., Bala-Mohan, S., Choli, D., Zahn, H. and Brandenburg, D. (1989) Biol. Chem. Hoppe-Seyler 370, 251–258.
[9] Fabry, M., Schaefer, E., Ellis, L., Kojro, E., Fahrenholz, F. and Brandenburg, D. (1992) J. Biol. Chem. 267, 8950–8956.
[10] Kurose, T., Pashmforoush, M., Yoshima, Y., Carroll, R., Schwartz, G.P., Burke, G.T., Katsoyannis, P.G. and Steiner, D.F. (1994) J. Biol. Chem. 269, 29190–29197.
[11] Schaefer, E.M., Siddle, K. and Ellis, L. (1990) J. Biol. Chem. 265, 13248–13253.
[12] Kristensen, C., Wiberg, F.C., Schäffer, L. and Andersen, A.S. (1998) J. Biol. Chem. 273, 17780–17786.
[13] Kristensen, C., Wiberg, F.C. and Andersen, A.S. (1999) J. Biol. Chem. 274, 37351–37356.
[14] Gustafson, T.A. and Rutter, W.J. (1990) J. Biol. Chem. 265, 18663–18667.
[15] Andersen, A.S., Kjeldsen, T., Wiberg, F.C., Christensen, P.M., Rasmussen, J.S., Norris, K., Moller, K.B. and Moller, N.P.H. (1990) Biochemistry 29, 7363–7366.
[16] Kjeldsen, T., Andersen, A.S., Wiberg, F.C., Rasmussen, J.S., Schäffer, L., Balschmidt, P., Moller, K.B. and Moller, N.P. (1991) Proc. Natl. Acad. Sci. USA 88, 4404–4408.
[17] Schumacher, R., Mosthaf, L., Schlessinger, J., Brandenburg, D. and Ullrich, A. (1991) J. Biol. Chem. 266, 19288–19295.
[18] Schumacher, R., Soos, M.A., Schlessinger, J., Brandenburg, D., Siddle, K. and Ullrich, A. (1993) J. Biol. Chem. 268, 1087–1094.
[19] Soos, M.A., Siddle, K., Baron, M.D., Heward, J.M., Luzio, J.P., Bellatin, J. and Lennox, E.S. (1986) Biochem. J. 235, 199–208.
[20] Zhang, B. and Roth, R.A. (1991) Proc. Natl. Acad. Sci. USA 88, 9858–9862.
[21] Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A. and Rutter, W.J. (1985) Cell 40, 747–758.
[22] Ullrich, A., Bell, J.R., Chen, E.Y., Herrara, R., Petruzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985) Nature 313, 756–761.
[23] Flores-Riveros, J.R., Sibley, E., Kastelic, T. and Lane, M.D. (1989) J. Biol. Chem. 264, 21557–21572.
[24] Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Cell 45, 721–732.
[25] Norrander, J., Kempe, T. and Messing, J. (1983) Gene 26, 101–106.
[26] Carter, P. (1987) Methods Enzymol. 154, 382–403.
[27] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[28] Bebbington, C.R. and Hentschel, C.C.G. (1987) in: DNA Cloning (Glover, D., Ed.), Vol. III, pp. 163–188, IRL Press, Oxford.
[29] Harlow, E and Lane, D. (1988) Antibodies: a Laboratory Manual, pp. 139–243, Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY.
[30] Prigent, S.A., Stanley, K.K. and Siddle, K. (1990) J. Biol. Chem. 265, 9970–9977.
[31] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239.
[32] Gill, R., Wallach, B., Verma, C., Urso, B., Dewolf, E., Grotzinger, J., Murrayrust, J., Pitts, J., Wollmer, A., DeMeyts, P. and Wood, S. (1996) Protein Eng. 9, 1011–1019.
[33] Bayne, M.L., Applebaum, J., Underwood, D., Chicchi, G.G., Green, B.G., Hayes, N.S. and Cascieri, M.A. (1988) J. Biol. Chem. 264, 11004–11008.
[34] Jansson, M., Andersson, G., Uhlen, M., Nilsson, B. and Kordel, J. (1998) J. Biol. Chem. 273, 24701–24707.
[35] Zhang, W.G., Gustafson, T.A., Rutter, W.J. and Johnson, J.D. (1994) J. Biol. Chem. 269, 10609–10613.
[36] Taylor, R., Soos, M.A., Wells, A., Argyraki, M. and Siddle, K. (1987) Biochem. J. 242, 123–129.
[37] Tulloch, P.A., Lawrence, L.J., McKern, N.M., Robinson, C.P., Bentley, J.D., Cosgrove, L., Ivancic, N., Lovrecz, G.O., Siddle, K. and Ward, C.W. (1999) J. Struct. Biol. 125, 11–18.