Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 191-198

BINDING KINETICS OF MUTATED INSULIN RECEPTORS IN TRANSFECTED CELLS GROWN IN SUSPENSION CULTURE:

Application to the Tyr → Phe 960 insulin receptor mutant

Ronald M. Shymko[1], Noe S. Gonzales[1], Jonathan M. Backer[2], Morris F. White[2] and Pierre De Meyts[1,3]

[1]Department of Diabetes, Endocrinology and Metabolism and [3]Molecular Genetics, City of Hope National Medical Center, Duarte, CA 91010

[2]Research Division, Joslin Diabetes Center, Boston, MA 02215

Received August 25, 1989

SUMMARY Site-directed mutagenesis of the insulin receptor cDNA is now widely used to elucidate the role of various domains and residues of the receptor, particularly in order to examine the functional importance of the β chain - associated tyrosine kinase. However, little has been done to correlate the functional repercussions of such mutations with alterations in the complex insulin binding kinetics. This is due in part to the difficulty of conducting large scale experiments using transfected cells on culture dishes. In an effort to overcome this problem, we have developed a method for culturing Chinese hamster ovary (CHO) cells in suspension culture, which provides a large number of cells and obviates the need for enzymatic or mechanical detachment of cells. The feasibility of this approach is demonstrated in a detailed study of the kinetics of insulin binding to the Tyr → Phe 960 insulin receptor mutant.

Since several groups have successfully cloned the cDNA for the insulin receptor and expressed it in various cell types (1-3), site-directed mutagenesis has been widely used to probe the functional importance of various receptor domains, especially in the evaluation of the physiological role of the receptor β-subunit protein-tyrosine kinase (4-10). Only recently have investigators attempted to use site-directed mutagenesis to elucidate the structure-function relationships of the insulin-binding α subunit (11-16). Lacking in most of these studies has been a careful evaluation of the repercussions of receptor mutations on the details of insulin binding kinetics. Such a correlation between function and kinetics is essential since the complex kinetics of insulin binding most probably represents the interplay of several ligand-induced conformational changes (17), the understanding of which may provide considerable insight into the mechanisms of receptor activation. For example, it was recently shown that monoclonal antibodies that stabilize the slow-dissociating ("K_{super}") state of the insulin receptor (17-21) mimicked the biological effects of insulin without stimulating receptor autophosphorylation (19,21), and were potent downregulators

0006-291X/89 $1.50

of the receptors. These data suggested that the K_{super} state may have physiological relevance with respect to receptor transmembrane signalling and internalization.

One reason why studies of correlation between kinetics and function of mutated receptors have been scarce is due in part to the difficulty of conducting large-scale experiments using transfected cells on culture dishes, especially dissociation experiments that require a large dilution factor and rapid separation of bound and free hormone (17,22).

In an effort to overcome this problem, we have developed a method for culturing Chinese hamster ovary (CHO) cells (one of the cell types preferentially used for stable transfection experiments), in suspension culture, which provides a large number of cells and obviates the need for enzymatic or mechanical detachment of cells. To demonstrate the feasibility of this approach, we present here a detailed study of the kinetic properties of insulin receptors mutated at tyrosine 960[1] (changed to phenylalanine). This mutation had no effect on the insulin-induced autophosphorylation of the receptor, or on its tyrosine kinase activity *in vitro*, but the mutated receptor failed to transmit the insulin signal as shown by a lack of insulin stimulation of glycogen synthetase, amino acid uptake and thymidine incorporation in transfected cells (23).

MATERIALS AND METHODS

Porcine insulin was purchased from Sigma (Batch #46F-0256; activity 26.3 U/mg).

[^{125}I-Tyr-A14]-insulin was prepared by iodination with the stoichiometric chloramine-T method and purified by HPLC using a RP-1/C18 reverse phase system (24). The precipitability of the tracer in 5% TCA was over 95%.

Cell culture. Cells transfected with normal or mutant forms of the human insulin receptor were grown in suspension in 250 ml Erlenmeyer flasks in 150 ml of McCoy's 5a media plus 15% fetal calf serum and 1.7 mg/ml G418. The cells were kept suspended using an orbital shaker at a setting of 140 rpm. Cell lines were maintained by splitting the culture 1:3 every second day into fresh media. For experiments, cultures were inoculated from a well-growing flask and used at 2 days post sub-culture.

Steady state binding studies. CHO cells (10^6 cells/ml) were incubated in 5 ml polystyrene tubes with ^{125}I-insulin (10 pM) for 150 min. at 15°C in buffer containing HEPES 100 mM; NaCl 120 mM; KCL 5 mM; Mg_2SO_4 1.2 mM; Na acetate 15 mM; glucose 10 mM; EDTA 1 mM; BSA 1% pH 7.6; in the presence of increasing concentrations of unlabeled insulin (0-10 μg/ml, i.e., 0-1.7 μM). Total volume was 0.5 ml. At the end of incubation, duplicate 200 μl aliquots from each tube were deposited on top of 200 μl ice cold buffer in 0.5 ml polypropylene microfuge tubes, which were spun for 60-90 sec. in a Beckman Microfuge. The supernatants were aspirated and the tips containing the pellets cut and counted for 10 min. in a γ-counter with 85% efficiency. The radioactivity in the pellets (minus the radioactivity observed in the presence of 10 μg/ml unlabeled insulin) was taken as specific binding. The bound/total radioactivity was plotted as a function of unlabeled insulin concentration (competition curves). The competition data

[1] Using the numbering system of Ullrich *et al.*

were fitted using proprietary programs on a Sun workstation to a model assuming negatively cooperative binding, with equilibrium dissociation constant K_d varying with receptor occupancy according to the relation

$$Kd = K_{de} + (B/Ro)\ (K_{df} - K_{de})$$

when B = bound insulin concentration, Ro = total receptor concentration, and K_{de} and K_{df} are the effective equilibrium dissociation constants near zero receptor occupancy and full occupancy, respectively (25).

This model does not take into account the "K_{super}" state, but since we showed recently that inclusion of the "K_{super}" state in the equilibrium analysis (26) does not change the shape of the competition curve or Scatchard plot (Shymko, R. and De Meyts, P. manuscript in preparation), we used the simpler model here for comparison purposes.

The specific binding data were also plotted as bound/free radioactivity, as a function of specifically bound (labeled plus unlabeled) insulin (Scatchard plots), and the theoretical curve corresponding to the parameters fitted above was superimposed on the experimental points.

Dissociation kinetics. The kinetics of dissociation of the ^{125}I-insulin from CHO cells was studied as previously described for IM-9 lymphocytes (17,21). The cells were washed twice, then resuspended at 1-1.5 X 10^7 cells/ml in buffer containing 100 mM hepes pH 7.6, 120 mM NaCl, 1.2 mM Mg_2SO_4, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose and 1% bovine serum albumin. Cells were incubated with [^{125}I-Tyr-A14]-insulin (10 pM) for 1 hour at 15°C. The cells were then centrifuged at 4°C and resuspended in the same volume of fresh Hepes buffer at 4°C. Dissociation was then initiated by a 40-fold dilution of 50 μl aliquots into two duplicate sets of 5 ml polyethylene tubes containing respectively either 2 ml Hepes buffer at 4°C or 15°C ("dilution alone"), or buffer with 1.7 nM unlabeled insulin ("dilution + insulin"). Appropriate sets of tubes were centrifuged at 4°C at the indicated time points for 2 min. at 1000 rpm in a Beckman GPR centrifuge, the supernatants aspirated and the pellets counted and expressed as a fraction of the radioactivity bound at time 0 (measured in duplicate 50 μl aliquots centrifuged immediately after resuspension and before dilution).

RESULTS

Growth of CHO cells in suspension (Figure 1)

The CHO cells adapted easily to growth in suspension culture. With an initial inoculum of 1 X 10^5 cells/ml and no change of media, CHO cell number doubled approximately every 24 hr. for 3 days and slowed thereafter as cell density increased to a plateau of 1 - 1.3 X 10^6 cells/ml. The growth curves for the CHO cells transfected with native insulin receptor cDNA (CHO-HIR) and those transfected with the Tyr → Phe 960 mutant receptor (CHO-F960) were identical. Based on these observations, the cell lines were maintained by splitting the culture 1:3 every second day into fresh media, and used 2 days post sub-culture for experiments.

Steady-state binding

Competition curves for CHO cells transfected with both native and mutated insulin receptor were identically shaped and spanned 3-4 log units, indicating complex binding. A typical experiment is shown in Figures 2a and b. The data fitted a negatively cooperative model (25) and, when transformed into Scatchard coordinates, demonstrated curvilinear plots (Figure 2c and d). The K_{de} values

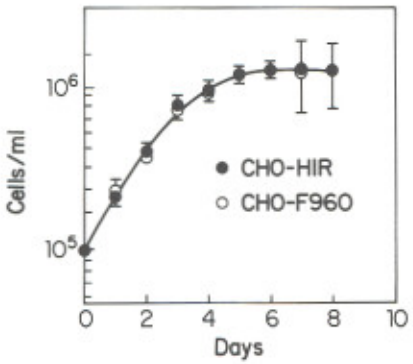

Figure 1. Growth of transfected CHO-HIR (●) and CHO-F960 (o) cells in suspension culture. The cells were cultured as explained in Materials and Methods. At the indicated times, an aliquot of cells was counted with a ZH Coulter counter.

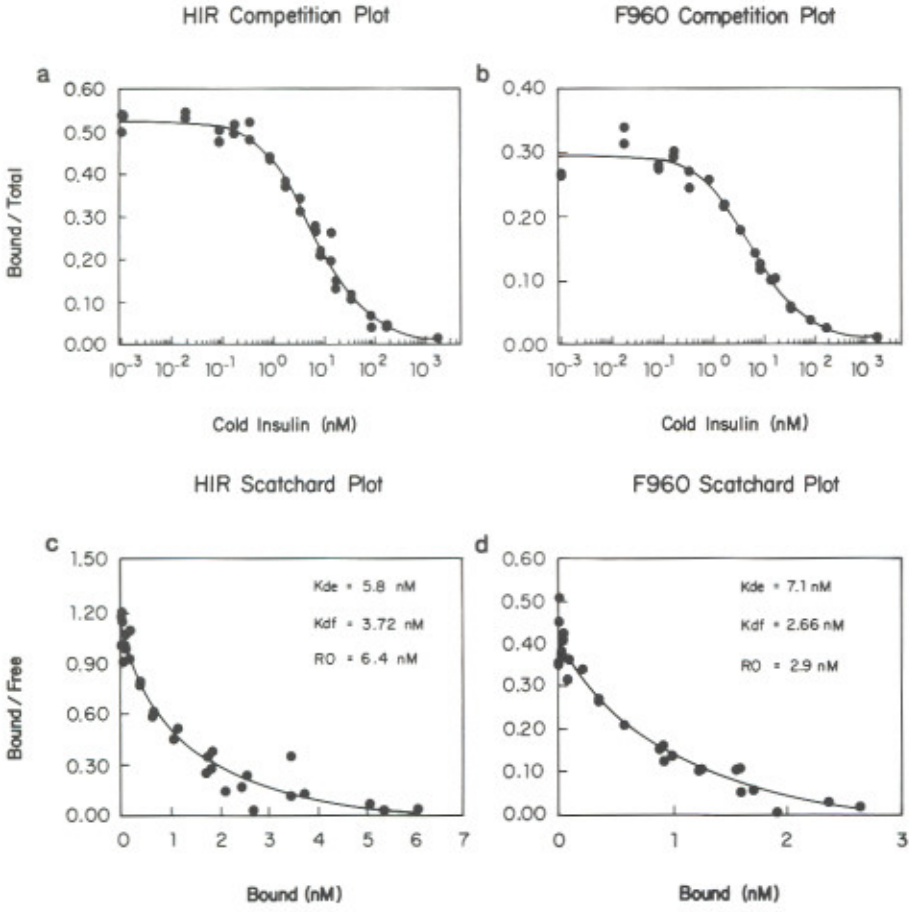

Figure 2. Steady-state binding of ^{125}I-insulin to CHO-HIR and CHO-F960 cells. Competition studies (top) were performed and Scatchard plots (bottom) were constructed and analyzed as explained in Materials and Methods.

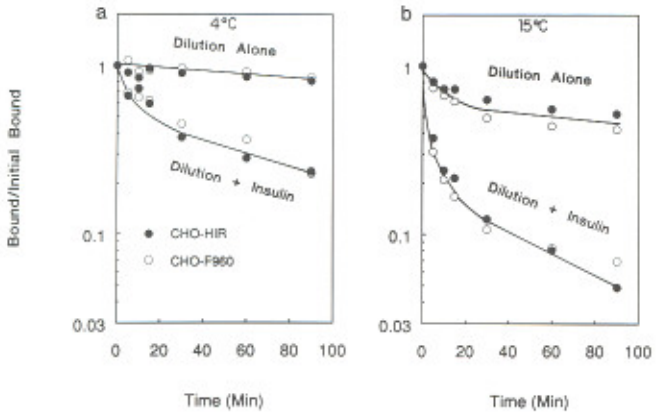

Figure 3. Kinetics of dissociation of ^{125}I-insulin from CHO-HIR and CHO-F960 cells at 4°C (left) and 15°C (right), by dilution in buffer alone or in buffer containing 1.7 X 10^{-7} M unlabeled insulin (see Materials and Methods for details).

for native and mutant receptors were respectively 5.8 and 7.1 nM, and the K_{dr} values 37.2 and 26.6 nM. The R_o value was 6.4 nM or ~384,000 receptors/cell for the native receptor versus 2.9 nM or ~174,000 receptors/cell for the mutant reflecting the usual variability in receptor expression between different transfections. The average fitted parameter values from seven different experiments were, for the cell lines transfected with native and mutant receptor respectively: Kde = 4.95 ± 4.55 nM and 5.21 ± 2.83 nM (not statistically different); Ro = 3.95 ± 3.22 nM and 1.78 ± 1.23 nM.

Dissociation kinetics

The dissociation rate of ^{125}I-insulin from both CHO-IR and CHO-F960 cells was markedly accelerated by unlabeled insulin at both 4°C and 15°C (Figure 3a and b) as previously demonstrated with native insulin receptors on other cell types (22,17) as well as with purified receptors (27). The dissociations were not first-order and slowed down with time in all cases. The dissociation curves obtained with the mutant receptor were clearly superimposable to those obtained with the native receptor and therefore were not submitted to more detailed quantitative analysis.

DISCUSSION

We have demonstrated the feasibility of adapting CHO cells to suspension culture with good yields. This method has two important advantages for the study of ligand binding to mutated receptors:

1) Large numbers of transfected cells can be obtained in a short period of time.

2) Binding studies can be performed directly in suspension without the potential for damage to cell surface receptors associated with enzymatic or mechanical detachment of cells from culture dishes. We have applied first this method to the study of the binding kinetics of the insulin receptor bearing a mutation of Tyr 960→ Phe, a mutation which markedly impairs signal transmission but not the autophosphorylation or phosphotransferase activity of the receptor (23).

We show here that there is no significant difference in the detailed kinetic and equilibrium binding characteristics of mutated versus normal insulin receptors expressed on the surface of transfected cells[2]. In particular, the negative cooperativity is intact as shown by the curvilinear Scatchard plots and the same degree of acceleration of dissociation of ^{125}I-insulin by unlabeled insulin; the presence of the so-called "K_{super}" state (17) is attested by the existence of a slow dissociating compartment with identical slope in both native and mutated receptors.

These results suggest that the signal transmission defect caused by the F960 mutation does not result from a global conformational change in the receptor affecting the insulin binding site or the site-site interactions (both negative and positive). The data support the more plausible conclusion (23) that the mutation alters locally the receptor β-chain's interaction with intracellular effector molecules or substrates mediating insulin action, as also suggested by the inability of the mutated receptor to phosphorylate pp185 in whole cells (23).

The method described here should be most valuable for studies in progress of the kinetic binding properties of insulin receptors mutated in the α-chain, which contains the insulin binding site.

ACKNOWLEDGMENTS

We are grateful to Axel Ullrich for providing the receptor cDNAs. Wanda Mutter provided excellent secretarial assistance. This work was supported by a grant from the American Diabetes Association, California Affilitate, to P.D.M.,

---

[2] We were somewhat surprised by the relatively low affinity of both the normal and mutated receptor transfects (~5 nM for K_{de} evaluated by computer fitting or 2.6 - 3.5 nM for the ED_{50} from competition curves). These values are lower than that reported by Ebina *et al.* (28) who show an ED_{50} around 1 nM (Figure 4 of Reference 28) for their insulin receptor in transfected CHO cells. This difference was not due to the suspension cultures since a similar K_d was found in the Boston laboratory for competition curves using culture dishes. Further studies are in progress in this and other (14) laboratories to evaluate potential affinity differences between the two natural variants (1,2) of the insulin receptor transfected in different cell types. Another possibility to be investigated is the ability of both receptor types to form hybrid molecules with IGF I receptors, the amount of which might then depend on the proportion of each receptor present in different cell types.

and NIH grant DK 38712 to M.F.W. M.F.W. is a Scholar of the Pew Foundation, Philadelphia. J.M.B. is recipient of a NRSA Fellowship DK 08126.

REFERENCES

1. Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J. Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grünfeld, C., Rosen, O.M., and Ramachandran, J. (1985) *Nature*, **313**,756-761.
2. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiaz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A., and Rutter, W.J. (1985) *Cell*, **40**,747-758.
3. Whittaker, J., Okamoto, A.K., Thys, R., Bell, G.I., Steiner, D.F., and Hoffmann, C.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,5237-5241.
4. Chou, C.K., Dull, T.J., Russel, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O.M. (1987) *J. Biol. Chem.*, **262**,1842-1847.
5. Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A., and Rutter, W.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,704-708.
6. Mc Clain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ullrich, A., and Olefsky, J.M. (1987) *J. Biol. Chem.*, **262**,14663-14671.
7. Ullrich, A., Riedel, H., Yarden, Y., Coussens, L., Gray, A., Dull, T., Schlessinger, J., Waterfield, M.D., and Parker, P.J. (1986) Cold Spring Harbor Symposia on Quantitative Biology, Vol. LI,713-724.
8. Ellis, L., Morgan, D.O., Clauser, E., Edery, M., Jong, S.M., Wong, L.H., Roth, R.A., and Rutter, W.J., (1986) Cold Spring Harbor Symposia on Quantitative Biology, Vol. LI,773-784.
9. Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A., and Rutter, W.J. (1986) *Cell*, **45**,721-732.
10. Morgan, D.O., and Roth, R.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,41-45.
11. De Meyts, P., Gu, J.-L., Shymko, R.M., Bell, G.I., and Whittaker, J. (1988) *Diabetologia*, **31**,484A, Abstract 127.
12. Whittaker, J., Thys, R., and De Meyts, P. (1988) *Diabetologia*, **31**,559A, Abstract 547.
13. Yip, C.C., Hsu, H., Patel, R.G., Hawley, D.M., Maddux, B.A., and Goldfine, I.D. (1989) *Biochem. Biophys. Res. Commun.*, **157**,321-329.
14. Mc Clain, D., Mosthaf, L., and Ullrich, A. (1989) *Diabetes*, **38**,Suppl. 2:1A, Abstract 1.
15. Rafaelof, R., Patel, R.G., Yip, C.C. and Hawley, D.W. (1989) *Diabetes*, **38**,Suppl. 2:1A, Abstract 2.
16. Kadowaki H., Kadowaki, T., Marcus-Samuels, B. Cama, A., Rovira, A., and Taylor, S.I. (1989) *Diabetes* **38**,Suppl. 2:2A, Abstract 8.
17. Gu, J.L., Goldfine, I.D., Forsayeth, J.R., and De Meyts, P. (1988) *Biochem. Biophys. Res. Commun.*, **150**,694-701.
18. Soos, M.A., Siddle, K., Baron, M.D., Heward, J.M., Luzio, H.P., Bellatin, J., and Lennox, E.S. (1986) *Biochem. J.*, **235**,199-208.
19. Forsayeth, J.R., Montemurro, A., Maddux, B.A., De Pirro, R., and Goldfine, I.D. (1987) *J. Biol. Chem.* **262**,4134-4140.
20. Forsayeth, J.R., Caro, J.F., Sinha, M.K., Maddux, B.A., and Goldfine, I.D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,3448-3451.
21. Soos, M.A., O'Brien, R.M., Brindle, N.P.J., Stigter, J.M., Okamoto, A.K., Whittaker, J. and Siddle, K. (1989) *Proc. Natl. Acad. Sci.*, **86**,5217-5221.
22. De Meyts, P., Roth, J., Neville, D.M., Jr., Gavin, J.R. III, and Lesniak, M.A. (1983) *Biochem. Biophys. Res. Commun.*, **66**,1118-1126.
23. White, M.F., Livingston, J.N., Backer, J.M., Lauris, V., Dull, T.J., Ullrich, A., and Kahn, C.R. (1988) *Cell*, **54**,641-649.
24. Markussen, J., and Larsen, U.D. (1980) In *"Insulin: chemistry, structure and function of insulin and related hormones* (D. Brandenburg and A. Wollmer, Eds.), pp. 161-168. De Gruyter, Berlin.

25. De Meyts, P., and Waelbroeck, M. (1978) In "*Membrane Receptors*", Proceedings 11th FEBS Meeting, Copenhagen, Pergamon Press, p. 319-323.
26. Shymko, R.M., Gu, J.L., Goldfine, I.D., and De Meyts, P. (1988) The Endocrine Society, 70th Annual Meeting, Abstracts, p. 241, Abstract 884.
27. Wang, C.C., Goldfine, I.D., Fujita-Yamaguchi, Y., Gattner, H.G., Brandenburg, D., and De Meyts, P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**,8400-8404.
28. Ebina, Y., Edery, M., Ellis, L., Standring, D., Beaudoin, J., Roth, R.A., and Rutter, W.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**,8014-8018.