The Binding Sites of Insulin-like Growth Factor I (IGF I) to Type I IGF Receptor and to a Monoclonal Antibody
MAPPING BY CHEMICAL MODIFICATION OF TYROSINE RESIDUES*

Peter Maly and Christine Luthi
From the Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

The surface topography of IGF I (insulin-like growth factor I) was investigated by chemical modification of amino acid residues in free IGF I and bound to type I IGF receptor or to monoclonal antibody MAB43. Tyrosine residues were modified either by chloramine-T or lactoperoxidase catalyzed iodination. In the free IGF I molecule, all 3 tyrosine residues, A19 (Tyr-60), B25 (Tyr-24), and C2 (Tyr-31), were iodinated. Monoclonal antibody MAB43 protected IGF I against modification at tyrosine residue A19, and in the type I IGF receptor-IGF I complex, all 3 tyrosine residues were shielded against iodine incorporation. These results allow the prediction of the binding domains in the IGF I molecule. The minimal receptor binding site in IGF I would include amino acid residues B25 to C2 and, possibly, the C-terminal part of the A-domain with tyrosine residue A19.

Binding of growth factors to cellular receptors is the first step in the signal transduction leading to cell division. One of these growth factors, insulin-like growth factor I (IGF I) is homologous in sequence to insulin (Rinderknecht and Humbel, 1978a, 1978b). IGF I preferentially binds to the type I IGF receptor (Nissley and Rechler, 1985, for review), which itself shows structural homology to the insulin receptor (Ulrich et al., 1986). The crystal structure of insulin has been determined (Blundell et al., 1971) and a three-dimensional model of IGF I, based on the insulin structure, could be predicted by computer-assisted model building (Blundell et al., 1978). The putative receptor binding region in the insulin molecule has been characterized by correlating binding data obtained from insulin receptor bound with insulin of different species (Blundell and Humbel, 1986, for review), for insulin mutants (Shoelson et al., 1983), or for chemically modified insulins (Gammeltoft, 1984, for review).

Chemical modification of amino acid residues is a powerful tool that can be used to probe the surface topography of proteins; the modified amino acid residues must, however, be identifiable (Bosshard, 1979, for review). In the type I IGF receptor-IGF I complex, the receptor may protect certain amino acid residues of IGF I from modification compared to free IGF I. This difference in modification can give us the first approximation of a growth factor's interacting domain that could be involved in the signal transduction of the growth signal to the cellular receptor.

Only pico-grams of purified proteins can be obtained conveniently in the case of growth factor receptors like the type I IGF receptor (Maly and Lüthi, 1986a). This limits the amount of receptor-IGF I complex available for analysis. Consequently, a modification reaction with a radioactive reagent had to be chosen, yielding a well-defined chemical product. The labeled amino acid residue must be unambiguously identifiable by peptide mapping and sequential Edman degradation. Iodination of tyrosine residues results in a stable product without any side reactions which would disturb tryptic digestion and sequential degradation of peptides (Maly et al., 1983). This method, therefore, meets all requirements.

The identification of amino acid residues in the IGF I molecule, which are accessible to iodination, catalyzed either by chloramine-T or lactoperoxidase, is described in this paper. Tyrosine residues of IGF I, protected from iodination in the monoclonal antibody MAB43-IGF I complex (epitope mapping), and type I IGF receptor-IGF I complex (receptor binding site mapping) are identified.

EXPERIMENTAL PROCEDURES

Materials—IGF I (preparations 1/3 or 1/4) was purified from human serum (Rinderknecht and Humbel, 1978) and labeled with Na'111 by the chloramine-T method (to approximately 80 Ci/g) (Zapf et al., 1981). The monoclonal antibody MAB43 was purified on DE52-cellulose (Whatman, United Kingdom) (Läubli et al., 1982). Type I IGF receptor from human placenta was purified as described previously (Maly and Lüthi, 1986a), with the following modifications: 0.6% (w/v) n-octyl-β-D-glucopyranoside, instead of Triton X-100, was used in the elution buffer during the IGF I-affinity chromatography; the receptor preparation was concentrated using Centricon 30 miniconcentrators (Amicon, Denvers, MA).

Complex Formation and Iodination Reaction—Binding assays were used to quantify the amounts of either type I IGF receptor or monoclonal antibody MAB43 (Maly and Lüthi, 1986b; Läubli et al., 1982), and the amount of binding sites was determined by the method of Scatchard (1949). A binding unit is defined as the amount of receptor or antibody necessary to bind one-half of the maximal bindable amount of 125I-IGF I in a binding assay. 500 units of type I IGF receptor, corresponding to approximately 20 pmol of IGF I binding sites (Kd = 50 pm), or 500 units of monoclonal antibody MAB43, corresponding to approximately 200 pmol of binding sites (Kd = 5 nm), respectively, were used for receptor/antibody-IGF I complex formation. Receptor/antibody and 20 pmol (170 ng) of IGF I were incubated in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.6% n-octyl-β-D-glucopyranoside in a volume of approximately 200 μl. The iodination reaction was carried out in the presence of 20 μM NaI (unlabeled) and 0.1 mCi Na'111, catalyzed by a chloramine-T concentration of 0.002% (w/v). The reaction was arrested by the addition of sodium metabisulfite to a final concentration of 0.02% (w/v). In the control experiments, 50 units of receptor/antibody preparation in the presence of 20 pmol of IGF I, or IGF I alone, were treated under the same conditions. Antibody-IGF I complex was also iodinated with the Enzymobead lactoperoxidase/glucose oxidase system (BioRad,
catalog no. 170-6001) (cf. Hubbard and Cohn, 1972, Wower et al., 1983). The iodination was initiated by the addition of 1/3-d-glucose to a final concentration of 0.2% (w/v). The reaction was terminated by the addition of sodium azide after incubation at room temperature for 30 min.

Analysis of the iodination products was performed on 5–20% gradient polycrylamide slab gels using the Laemmli system (1970). The gels were stained with Coomassie Blue and subsequently autoradiographed.

**Isolation of Iodinated IGF I**—The receptor/antibody-IGF I complexes, or free IGF I, were immediately desalted on Sephadex G-25 after the iodination reaction (2-ml syringes, in 0.1 M sodium phosphate buffer, pH 7.4, 0.1% Triton X-100). IGF I was extracted from the desalted complexes by acidification, with acetic acid, to pH 2.8, and, after an incubation time of 15 min, the dissociated components were loaded on a Sep-Pak C18 cartridge (Waters Associates, Milford, MA), according to the manufacturer's protocol. IGF I was eluted from the Sep-Pak cartridge with 40% acetonitrile in 0.1% trifluoroacetic acid and lyophilized. IGF I was finally purified by HPLC on an ODS column (Brownlee, Santa Clara, CA). The column was developed with a linear acetonitrile gradient (30–70%, flow rate 1 ml/min) in 0.1% trifluoroacetic acid and lyophilized. IGF I was finally purified by HPLC on an ODS column with a linear acetonitrile gradient (30–70%, flow rate 1 ml/min) in 0.1% trifluoroacetic acid. The tyrosine residues by N-terminal sequential degradation, according to the procedure of Chang et al. (1978) and Wower et al. (1983). The products were extracted with 0.5 ml ethyl acetate, dried, and identified on thin-layer plates. The plates were developed in the one-dimensional system toluene:in-hexane:acetic acid (21:1), autoradiographed, and compared with mono-iodinated and -iodinated tyrosine standard products (Sigma).

**RESULTS**

**Iodination of IGF I**—The complex between IGF I and the type I IGF receptor or monoclonal antibody MAB433 was preformed by incubation of the purified proteins in a 1:1 ratio. The amount of receptor or antibody necessary was determined by binding analysis.

Initially polyacrylamide gel electrophoresis trials with the IGF I-type I IGF receptor complex indicated that no incorporation of iodine into the IGF I molecule was detectable after the lactoperoxidase-induced reaction. In the case of monoclonal antibody-IGF I complex, however, the same tyrosine modification for IGF I was observed, either by iodination with lactoperoxidase or chloramine T. We therefore used the iodination condition is not the limiting factor.

**Analysis of Iodinated Tyrosine Residues in IGF I**—The iodinated complexes and control IGF I, respectively, were extracted at pH 2.8, and IGF I was purified on Sep-Pak reversed phase cartridges and HPLC. The purified IGF I fractions were then S-carboxymethylated, desalted on Sep-Pak C18 cartridges and digested with trypsin. A reproducible peptide map was obtained by adding bovine serum albumin as a carrier protein to the carboxymethylation reaction. Fig. 2 exhibits the corresponding radioactive profiles from the high performance liquid chromatograms of the tryptic digests. The peptides were subsequently identified by the release of the radioactive label during Edman degradation, presented in Fig. 3, followed by thin-layer chromatography to identify the radioactive tyrosine products (see "Experimental Procedures").

IGF I, as control, (lanes e and f in Fig. 1) produced two radioactive peptides (Fig. 2C), which were identified by Edman degradation (Fig. 3), and could be fitted to the sequence of IGF I (Fig. 4). Peak A represents the tryptic peptide T5, with Tyr-60 (A19), and peak B peptide T2, with Tyr-24 (B25) and Tyr-31 (C2). The elution profile of iodinated peptides T2 and T5 from the HPLC was in agreement with unmodified...
IGF I Binding Site to Type I IGF Receptor

**FIG. 2.** Radioactive profile of the HPLC analysis of tryptic peptides of iodinated IGF I. A, type I IGF receptor-IGF I complex (Fig. 1, lanes a and b); B, monoclonal antibody MAB43-IGF I complex (Fig. 1, lanes c and d); and C, control IGF I (Fig. 1, lane e). Solid lines mark the profile of receptor/antibody-IGF I complexes; dotted lines mark the profile of controls with a 10-fold excess of IGF I over receptor/antibody. The gradient of acetonitrile (30–60%, see "Experimental Procedures") was applied between fractions 20–100. Peaks A, B, B', and B" are derived from IGF I (see text), and peaks marked with (*) do not fit to the sequence of IGF I.

**FIG. 3.** N-terminal sequential degradation of peptide peaks A, B, and B' from the HPLC analysis of Fig. 2 are presented. The \(^{125}\)I radioactivity released at each cycle is given in counts/min (% of total before cycle 1), and the tryptic peptides of IGF I are fitted to the sequence (Rinderknecht and Humbel, 1978a).

Tryptic peptides of IGF I (Honegger and Humbel, 1986). The heterogeneous N terminus of peptide T5, Arg or Leu, was also detected in unmodified tryptic digests of IGF I and is easily explained as a tryptic cleavage product of the preleading Arg-Arg sequence (see Fig. 4). Furthermore, analysis of the radioactive products from Edman degradation on thin-layer chromatography showed clearly that the radioactivity detected in cycle 3 of peptide A (Fig. 3) is not identical with mono- or di-iodinated tyrosine products. The same results were obtained for the peptides from the receptor/antibody complexes (Fig. 1, lanes c and d, and lanes e and f), where IGF I was present in a 10-fold excess during the modification reaction (Fig. 2, A and B).

In the peptide map of IGF I, isolated from the antibody-IGF I complex (lanes c and h in Fig. 1 and Fig. 2B), peak A, representing peptide T5, was missing. In addition to peak B, the peaks B' and B" appeared in some cases (Fig. 2, A and B). Sequential degradation of these materials showed them to be identical to peptide T2. Thin-layer chromatography of the Edman degradation products from peak B' identified in cycle 3, mono-iodinated, and in cycle 10, di-iodinated, tyrosine products. For peak B" both tyrosine residues were found to be di-iodinated as was found in the case of peak B. The other peaks marked by an asterisk (*) in Fig. 2B could not be fitted to the IGF I sequence after sequential degradation and were interpreted as contaminants from the monoclonal antibody preparation. These results show that tyrosine residue A19 of IGF I is protected against modification in the antibody-IGF I complex.

IGF I isolated from the type I IGF receptor-IGF I complex (Fig. 1, lanes a and f) did not produce a significant amount of \(^{125}\)I-labeled peptides, as shown in Fig. 2A. These results indicate complete protection of all 3 tyrosine residues, B25, C2, and A19, of IGF I by the type I IGF receptor against chemical modification with iodine.

**FIG. 4.** Model and sequence of IGF I molecule. Protected tyrosine residues in IGF I are marked as listed below. The tryptic peptides T2 and T5 are underlined in the sequence of IGF I (Rinderknecht and Humbel, 1978a) and tyrosine residues are marked by boxes. Tyrosines in the three-dimensional model of IGF I (Honegger, 1985) are shown by their side chains. The solid box/bold ring indicates tyrosine A19, which is protected from iodination when in the receptor/antibody complex. The dashed boxes/shaded rings indicate tyrosines B25 and C2, which are protected against modification only in the type I IGF receptor complex.
DISCUSSION

Free IGF I was iodinated either with chloramine T, or with the combined lactoperoxidase/glucose oxidase immobilized enzyme system (Hubbard and Cohn, 1972) (data not shown), in order to monitor the accessible tyrosine residues on the surface of the free IGF I molecule.

An important precondition in the differential modification method is the prevention of chemically induced distortions in protein structures, which could possibly mimic a nonexistent interacting domain.

All the experiments presented in this paper, the type I IGF receptor-IGF I complex was only available in picomolar amounts (cf. Introduction). Preliminary tests with receptor-IGF I complex indicated that the molar concentration of iodine in the reaction mixture had to be adjusted to 20 μM for reproducible tyrosine modification. This resulted in lower specific radioactivity and forced us to compromise the degree of iodination per molecule of complex to locate the modified tyrosine residues unambiguously. Incorporation of approximately 10 iodine molecules/molecule of type I IGF receptor-IGF I complex was necessary under these conditions.

Normally the modification reaction is limited to statistically less than 1 modified residue/molecule of protein complex. Several control experiments justify our procedure. Differential modification of the antibody MAB43-IGF I complex with a degree of iodine incorporation of 10, respectively, 0.2–0.9 molecules/complex, leads to the same structural result. Furthermore, in a kinetic study no qualitative changes in the iodine incorporation rate into tyrosine residues of IGF I were observed during a 5- to 30-min interval. In the case of lower iodine incorporation (data not shown) into the antibody-IGF I complex, or into control IGF I, two different peaks were detected for peptide T5 of IGF I (mono- and di-iodinated Tyr-60, respectively); peptide T2 of IGF I was identified in four different peaks (data not shown), representing the single iodinated species in Tyr-24, or -31, respectively, and the double iodinated peptide (Tyr-24 and -31), which was herein identified (Fig. 2B and Fig. 3, peaks B and B').

CONCLUSIONS

Examination of the model of IGF I which is based upon model-building operations, shown in Fig. 4 (Blundell et al., 1978; Honegger, 1985), reveals that 2 tyrosine side chains are located completely free on the surface of IGF I (B25 and C2) and that 1 tyrosine (A19) is slightly concealed in the C-terminal region of the molecule. All 3 tyrosine residues are accessible for iodination reaction in the free state of IGF I. The fact that tyrosine residue A19 was also iodinated by the lactoperoxidase-catalyzed system, which is highly dependent on the immediate environment of the tyrosine residue (Bayse et al., 1972), suggests a relatively free accessibility of this amino acid residue. The tyrosine residue A19 was isolated against iodination in the case of the monoclonal antibody MAB43-IGF I complex. This result may reflect a contribution of this tyrosine residue to the epitope. One cannot exclude the possibility that the antibody binding to IGF I induces a more rigid conformation, as seen in the model (Fig. 4), protecting tyrosine residue A19 against modification. One can, however, conclude that the end of the B-domain and part of the C-domain of IGF I are not involved in antibody MAB43 binding. This is evidenced by the unhindered iodination of tyrosine residues B25 and C2 in the complex.

The homologies between insulin and IGF I, as well as insulin receptor and type I IGF receptor, respectively, suggest an analogous receptor binding site. A series of binding experiments with modified insulin molecules and insulin mutants (Gammeltoft, 1984, for review) clearly showed that the amino acid side chains at the C-terminal part of B- and A-domains, as well as the N terminus of the A-domain, are essential for insulin receptor binding. Our results, showing high protection of the 3 tyrosine residues in IGF I through type I IGF receptor, agree perfectly with the predicted receptor binding site analogous to insulin. The tyrosine residue C2 is located at the same side of the molecule as residue B25 in the model of IGF I (Fig. 4). Position B24 to B26 is a conserved region of Phe-Tyr-Phe in the IGFs and Phe-Phe-Tyr in most insulins. Tyrosine residue A19 is conserved in all mutants known. Due to the protection of these tyrosine residues during the modification reaction of the receptor-IGF I complex, we predict a minimum binding site in IGF I covering the domain from residue B25 to C2 and possibly portions of the C-terminal part of the A-domain, including residue A19. One must restrict the interpretation of results from the protection of tyrosine A19; receptor binding could induce a more rigid conformation in the C-terminal part of the IGF I as it could be in the case of antibody-bound IGF I.

IGFs of different species with altered binding affinity to type I IGF receptor are poorly characterized (Zanger et al., 1987), and the sequences of bovine (Honegger and Humbel, 1986), mouse (Bell et al., 1986), and rat (Shimatsu and Rotwein, 1987) IGF I are too similar to serve in interpreting receptor binding data. Three different synthetic insulin-IGF I hybrids (King et al., 1982, and De Vroede et al., 1986) have been constructed and show in each case a significantly reduced receptor binding activity. These experiments suggest only that the C-terminal domain of IGF I (D-peptide) could be important for the selective binding to type I IGF receptor.

In our attempt to characterize the binding site between IGF I and the type I IGF receptor, the mapping of surface-located amino residues Asp and Glu (Bechold and Bosshard, 1985) and Lys (Rieder and Bosshard, 1980) would provide a more detailed profile of the interacting domain.

Acknowledgments—We thank Dr. R. H. Bosshard and Marc Yaffe for the critical reading of the paper. We are very grateful to Dr. R. Humbel for his continued support.

REFERENCES

Bayse, G. S., Michaels, A. W. & Morrison, M. (1972) Biochim. Biophys. Acta 284, 30–33
Bechold, R. & Bosshard H. R. (1985) J. Biol. Chem. 260, 5191–5200
Bell, G. I., Stempien, M. M., Fong, N. M. & Rall, L. B. (1986) Nucleic Acids Res. 14, 7873–7880
Blundell, T. L. & Humbel, R. E. (1980) Nature 287, 781–787
Blundell, T. L., Cuffield, J. F., Cufffield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Mercola, D. A. & Vijayan, M. (1971) Nature 231, 506–511
Blundell, T. L., Bedarkar, S., Rinderknecht, E. & Humbel, R. E. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 180–184
Bosshard, H. R. (1979) Methods Biochem. Anal. 25, 273–301
Chang, J. Y., Brauer, D. & Wittmann-Liebold, B. (1978) FEBS Lett. 93, 205–214
De Vroede, M. A., Rochler, M. M., Nisley, S. P., Ogawa, H., Joshi, S., Burke, G. T. & Katsuyamas, P. G. (1986) Diabetes 35, 355–361
Gammeltoft, S. (1984) Physiol. Rev. 64, 1291–1378
Hirs, C. H. W. (1967) Methods Enzymol. 11, 199–203
Honegger, A. (1985) Bovine Insulin-like Growth Factors I and II. Ph.D. dissertation, University of Zürich
Honegger, A. & Humbel, R. E. (1986) J. Biol. Chem. 261, 569–575
Hubbard, A. L. & Cohn, Z. A. (1972) J. Cell Biol. 55, 390–405
King, G. L., Kahn, C. R., Samuels, B., Danho, W., Bullesbach, E. E. & Gattner, H. G. (1982) J. Biol. Chem. 257, 10869–10873
Laemmli, U. K. (1970) Nature 227, 680–685
Laubli, U., Baier, W., Binz, H., Celio, M. R. & Humbel, R. E. (1986) FEBS Lett. 149, 109–112
Malý, P. & Lütich, G. (1986a) Biochem. Biophys. Res. Commun. 137, 695–701
Maly, P. & Liithi, C. (1986b) Biochem. Biophys. Res. Commun. 138, 1257-1262
Maly, P., Wower, J., Zobawa, M. & Brimacombe, R. (1983) Biochemistry 22, 3157-3162
Nissley, S. P. & Rechler, M. M. (1984) Hormonal Proteins and Peptides, Vol. XII, pp. 128-195, Academic Press, Orlando, FL
Rieder, R. & Bosshard, H. R. (1980) J. Biol. Chem. 255, 4732-4739
Rinderknecht, E. & Humbel, R. E. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4379-4381
Rinderknecht, E. & Humbel, R. E. (1978a) J. Biol. Chem. 253, 2769-2776
Rinderknecht, E. & Humbel, R. E. (1978b) FEBS Lett. 99, 283-289
Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672
Shimatsu, A. & Rotwein, P. (1987) J. Biol. Chem. 262, 7894-7900
Shoelson, S., Haneda, M., Blix, P., Nanjo, A., Sanke, T., Inouye, K., Steiner, D., Rubenstein, A. & Tager, H. (1983) Nature 302, 540-543
Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2503-2512
Wower, J., Maly, P., Zobawa, M. & Brimacombe, R. (1983) Biochemistry 22, 2339-2346
Zangerl, L., Zapf, J. & Froesch, E. R. (1977) Acta Endocrinol. 114, 107-112
Zapf, J., Walter, H. & Froesch, E. R. (1981) J. Clin. Invest. 68, 1321-1330
Zumstein, P. P. & Humbel, R. E. (1985) Methods Enzymol. 109, 782-796
Zumstein, P. P., Liithi, C. & Humbel, R. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3169-3172