A monoclonal anti-peptide antibody reacting with the insulin receptor β -subunit

Characterization of the antibody and its epitope and use in immunoaffinity purification of intact receptors

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A mouse monoclonal antibody (CT-1) was prepared against the C-terminal peptide sequence of the human insulin receptor β -subunit (KKNRILTLPRSNPS). The antibody reacted with native human and rat insulin receptors in solution, whether or not insulin was bound and whether or not the receptor had undergone prior tyrosine autophosphorylation. The antibody also reacted specifically with the receptor β -subunit on blots of SDS/polyacrylamide gels. Preincubation of soluble receptors with antibody increased the binding of ^{125}I -insulin approx. 2-fold. The antibody did not affect insulin-stimulated autophosphorylation, but increased the basal autophosphorylation rate approx. 2-fold. The amino acid residues contributing to the epitope for CT-1 were defined by construction and screening of an epitope library. Oligonucleotides containing 23 random bases were synthesized and ligated into the vector pCL627, and the corresponding peptide sequences expressed as fusion proteins in *Escherichia coli* were screened by colony blotting. Reactive peptides were identified by sequencing the oligonucleotide inserts in plasmids purified from positive colonies. Six different positive sequences were found after 900 000 colonies had been screened, and the consensus epitope was identified as GRVLTLPRLS. Phosphorylation of the threonine residue within this sequence (corresponding to the known phosphorylation site Thr-1348 in the insulin receptor) decreased the affinity of antibody binding approx. 100-fold, as measured by competition in an e.l.i.s.a. Antibody CT-1 was used for immunoaffinity isolation of insulin receptor from detergent-solubilized human placental or rat liver microsomal membranes. Highly purified receptor was obtained in 60 % yield by binding to CT-1-Sepharose immunoabsorbent and specific elution with a solution of peptide corresponding to the known epitope. This approach to purification under very mild conditions may in principle be used with any protein for which an antibody is available and for which a peptide epitope or 'mimotope' can be identified.

INTRODUCTION

The insulin receptor has been well characterized as a heterotetrameric membrane glycoprotein, which possesses intrinsic ligand-stimulated tyrosine-specific protein kinase activity. However, many details of the structure, regulation and signalling mechanism of the receptor remain unclear (reviewed in Zick, 1989; Houslay & Siddle, 1989; Olefsky, 1990). There is thus a continuing need for reagents that facilitate the isolation of receptors or genetically engineered constructs for structural or functional studies.

Receptor antibodies have proved particularly valuable in this context, particularly for the specific immunoprecipitation of receptor from cell lysates, but also for bulk purification and for investigating mechanisms of activation [reviewed in Kahn *et al.* (1981), Roth & Morgan (1985) and Siddle *et al.* (1988)]. Polyclonal antibodies were obtained initially from patients with the autoimmune syndrome of type B insulin resistance (Kahn *et al.*, 1981), and subsequently by immunization of rabbits (Jacobs & Cuatrecasas, 1981). However, all such antisera are limited in availability, and potentially heterogeneous with regard to the affinity and fine specificity of antibody subpopulations. A substantial number of monoclonal antibodies have been produced, using intact receptor as immunogen (Kull *et al.*, 1983; Morgan & Roth, 1986; Soos *et al.*, 1986; Forsayeth *et al.*, 1987). Most of

these antibodies obtained in mice are specific for human insulin receptors and therefore not applicable to many animal models, especially the rat. It has sometimes been possible to show whether antibody binds to the α - or β -subunit of the receptor (Morgan & Roth, 1986; Soos *et al.*, 1986) and in a few instances to define 'linear' peptide epitopes (Prigent *et al.*, 1990). However, many epitopes for monoclonal antibodies are very conformationally dependent and it is difficult to define these precisely for a molecule of the size and complexity of the insulin receptor.

Antibodies reacting at known and predetermined sites may in principle be obtained by using as immunogens synthetic peptides conjugated to carrier proteins. However, anti-peptide antibodies frequently do not recognize native proteins, or at best do so only with low affinity, reflecting inaccessibility and/or conformation of specific sequences in the native structure compared with synthetic conjugate or free peptide. Experiences with insulin receptor peptides have been typical in this respect, although antibodies have been obtained for sites on both the α - and β -subunits which do recognize native receptor as well as reacting with denatured receptor on nitrocellulose blots (Herrera *et al.*, 1985, 1986; Grunfeld *et al.*, 1987; Baron *et al.*, 1989, 1990; Perlman *et al.*, 1989; Toyoshige *et al.*, 1989; Pessina *et al.*, 1989).

Both theoretical and empirical guidelines have been suggested for prediction of peptide sequences within complex proteins

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; IGF-I, insulin-like growth factor I; PEG, poly(ethylene glycol) 6000; CHO.T, Chinese hamster ovary cells transfected with human insulin receptor cDNA; KLH, keyhole limpet haemocyanin; DTT, dithiothreitol.

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which are likely to elicit antibodies reacting with native as well as denatured protein (Getzoff *et al.*, 1988). Sequences at the C-terminus are favoured in this context, as these are likely to be more accessible and less conformationally constrained than those in other parts of the molecule. In the case of the insulin receptor there is the additional advantage that the C-terminal sequence is conserved between species (Ullrich *et al.*, 1985; Ebina *et al.*, 1985; Flores-Riveros *et al.*, 1989; Goldstein & Dudley, 1990). We set out to obtain antibodies for this sequence which would be useful in the precipitation, purification and assay of both human and rodent insulin receptors. We report here the production and characterization of a mouse monoclonal anti-peptide antibody CT-1, which reacts with native and denatured receptors. We have further defined the amino acid residues contributing to the epitope for this antibody by screening a library of random peptides expressed as fusion proteins in bacteria. A purification protocol is described in which receptor is eluted from a CT-1 affinity adsorbent by using a solution of free peptide, illustrating an approach which should be generally applicable for immunoaffinity purification of proteins under mild conditions.

EXPERIMENTAL

Materials

Bovine insulin, aprotinin, bacitracin, antipain, phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM) and CNBr-activated Sepharose 4B were from Sigma Chemical Co., Poole, Dorset, U.K. Highly purified desamido-free bovine insulin and recombinant human insulin-like growth factor I (IGF-I) were gifts respectively from Dr. D. Brandenburg (Deutsches Wollforschungsinstitut, Aachen, Germany) and Dr. K. Scheibli (Ciba-Geigy, Basel, Switzerland). Radiochemicals (Na^{125}I , Cat. no. IMS 30 and [^{32}P]phosphate, PBS 11) were from Amersham International, Aylesbury, Bucks., U.K. Bovine γ -globulin and rabbit antisera specific for mouse immunoglobulin subclasses were from Miles Biochemicals, Slough, Bucks., U.K. Affinity-purified goat anti-rabbit immunoglobulin and goat anti-mouse immunoglobulin, coupled to horseradish peroxidase, were from Tago, Burlingame, CA, U.S.A. Nitrocellulose paper (0.2 μm pore size) was from Schleicher and Schuell, Dassel, Germany. Poly(ethylene glycol) 6000 (PEG) was from BDH Chemicals, Dagenham, Kent, U.K. Fetal bovine serum was from Northumbria Biologicals Ltd., Cramlington, U.K., and other cell culture reagents were from Gibco Ltd., Paisley, Scotland.

Chinese hamster ovary cells transfected with human insulin receptor cDNA, (CHO.T; Ellis *et al.*, 1986), were a gift from Dr. L. Ellis (Howard Hughes Medical Institute, Dallas, TX, U.S.A.). IM-9 lymphocytes were from Flow Laboratories, Irvine, Scotland. Balb/c mice, New Zealand White rabbits and Wistar rats were supplied by Central Biomedical Services, University of Cambridge, Cambridge, U.K. Normal human placenta was freshly obtained at delivery.

The peptides RILTLPRSNPS and LPRSNPS were kindly synthesized by Professor R. M. Denton (Department of Biochemistry, University of Bristol, Bristol, U.K.). The phosphopeptide RILT(PO_3)LPRSNPS, synthesized and purified as described (Andrews *et al.*, 1991), was a generous gift from Dr. D. M. Andrews (Glaxo Group Research, Greenford, Middx., U.K.).

Production of antibodies

The hexadecapeptide YKKNRILTLPRSNPS was synthesized using F-moc chemistry by Cambridge Research Biochemicals, Harston, Cambs., U.K. This peptide corresponds to the C-terminal 15 amino acids of the human insulin receptor β -subunit (Ullrich *et al.*, 1985), together with an *N*-terminal

tyrosine added to facilitate radiolabelling and conjugation to protein. Peptide was coupled to keyhole limpet haemocyanin (KLH) or BSA, at a weight ratio of 1:4, using bis-diazotolidine (Basiri & Utiger, 1972; Briand *et al.*, 1985).

Rabbits were injected at multiple subcutaneous sites with 2 mg of peptide-KLH or peptide-BSA conjugate in Freund's adjuvant (complete for first injection, incomplete for subsequent injections) at monthly intervals. Animals were bled from an ear vein 10 days after each booster injection.

Mice were injected subcutaneously at a single site with 0.5 mg of peptide-KLH conjugate in complete Freund's adjuvant, and boosted after 1 month with the same amount of conjugate in incomplete adjuvant. After a further 6 months, the mice were given four intraperitoneal injections of 0.5 mg of peptide-BSA conjugate in phosphate-buffered saline (PBS; 10 mM-sodium phosphate, pH 7.4 and 150 mM-NaCl) at monthly intervals. The mice were test-bled from the tail and selected animals given a final intravenous injection of peptide-KLH conjugate together with 1 mg of purified human placental insulin receptor in 0.9% NaCl (Fujita-Yamaguchi *et al.*, 1983). At 4 days after the final boost, mice were killed and spleen cells taken for fusion with mouse NS1 myeloma cells according to standard methods (Galfre & Milstein, 1981). The products of each fusion were distributed into the wells of four microtitre plates and culture supernatants were screened after 10 days using the ^{125}I -insulin-receptor coprecipitation assay described previously (Soos *et al.*, 1986). Antibody-secreting cells were cloned twice at limiting dilution using mouse peritoneal macrophages as feeder cells. Cloned cells were grown as ascites tumours in pristane-primed mice, and ascites fluid was collected containing specific monoclonal antibody at 1–2 mg/ml.

Antibody was routinely partially purified from ascites fluid by precipitation with 40% satd. $(\text{NH}_4)_2\text{SO}_4$, and dialysed against PBS. Isotyping was performed by dot-blot immunobinding assay (Beyer, 1984), using rabbit antisera specific for mouse IgG1, IgG2a, IgG2b, IgG3 and IgM subclasses.

Immunoabsorbents

Antibody CT-1 was purified by hydroxyapatite chromatography (Stanker *et al.*, 1985). Purified antibody was coupled to finely divided aminocellulose by diazotization (Hales & Woodhead, 1980) or to CNBr-Sepharose according to the manufacturer's instructions. The amounts of antibody coupled were approx. 200 $\mu\text{g}/\text{mg}$ of cellulose and 10 $\mu\text{g}/\text{mg}$ of Sepharose respectively. Sheep anti-mouse IgG immunoabsorbent was as previously described (Soos & Siddle, 1982).

Radiolabelling

Mono- ^{125}I -iodoinsulin (150–200 $\mu\text{Ci}/\text{mg}$) and ^{125}I -IGF-I (100 $\mu\text{Ci}/\text{mg}$) were prepared as described previously (Soos & Siddle, 1989), and [γ - ^{32}P]ATP was prepared by the method of Glynn & Chappell (1963).

Receptor-binding assays

Microsomal membrane fractions prepared from human placenta or rat liver as described by Fujita-Yamaguchi *et al.* (1983) were used as sources of receptor. Membranes were solubilized in 50 mM-Hepes, pH 7.6, containing 2% (w/v) Triton X-100 and proteinase inhibitors PMSF (1 mM), benzamidine (2.5 mM), pepstatin (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$), antipain (1 $\mu\text{g}/\text{ml}$), aprotinin (0.2 trypsin inhibitory unit/ml) and bacitracin (1 mg/ml). The assay buffer but not the solubilization buffer also contained NEM (1 mM). For some experiments, receptors were partially purified by chromatography on wheat germ agglutinin-Sepharose (Fujita-Yamaguchi *et al.*, 1983). Receptor preparations were preincubated with ^{125}I -insulin or ^{125}I -IGF-I for 16 h

at 4 °C before addition of antibody. Total and immunoprecipitable ^{125}I -ligand-receptor complexes were determined by PEG precipitation or binding to sheep anti-mouse IgG immuno-adsorbent (or Protein A-Sepharose) as previously described (Soos *et al.*, 1986; Soos & Siddle, 1989). Radioactivity was determined in an NE1600 γ -counter with an efficiency of 70 %.

Insulin binding to IM-9 cells was carried out as described by Roth *et al.* (1982).

Immunoblotting

Confluent CHO.T cells in a 80 cm² flask (approx. 10⁷ cells) were rinsed with PBS and solubilized by scraping into 1.5 ml of lysis buffer (50 mM-Hepes, pH 7.6, 1 % Triton X-100, 1 mM-PMSF, 1 mg of bacitracin/ml, 1 mM-EDTA, 10 mM-NaF, 1 mM-sodium vanadate) for 10 min. The lysate was sonicated for 1 min and centrifuged at 12000 *g* for 10 min. The supernatant was mixed with 3 $\times$ electrophoresis sample buffer including 300 mM-dithiothreitol (DTT), and electrophoresed across the whole width (20 cm) of an SDS/polyacrylamide gel (7.5 % polyacrylamide) (Laemmli, 1970). The gel was blotted on to nitrocellulose paper (Burnette, 1981) which was then incubated with a 2 % (w/v) suspension of dried milk powder in PBS for 1 h at room temperature. The blot was cut into 1 cm strips, which were incubated with rabbit antisera or mouse monoclonal antibodies and developed using peroxidase-conjugated second antibodies (Soos *et al.*, 1986).

Insulin-receptor autophosphorylation

Solubilized placental microsomal membranes were used as a source of receptor. Autophosphorylation reactions were carried out at 4 °C, after incubation with insulin or antibody, as previously described (O'Brien *et al.*, 1987). In experiments to investigate the effect of antibody on phosphorylation, the reaction was stopped by addition of 3 $\times$ electrophoresis sample buffer containing 300 mM-DTT, and samples were loaded directly on to SDS/polyacrylamide gels. In experiments to test immunoprecipitation of autophosphorylated receptor, the reaction was terminated by addition of 4 $\times$ stop solution (100 mM-Hepes, pH 7.6, 20 mM-EDTA, 4 mM-PMSF, 400 mM-NaF, 40 mM-sodium pyrophosphate, 20 % glycerol, 0.4 % Triton X-100). Samples (60 μl) from autophosphorylation reactions carried out in bulk were incubated for 1 h at 4 °C with antibody (10 μl of hybridoma culture supernatant, approx. 0.5 μg of antibody) before addition of 0.1 mg of sheep anti-mouse IgG immuno-adsorbent for 30 min. The adsorbent was pelleted by centrifuging at 2500 *g* for 10 min, and washed three times in 50 mM-Hepes, pH 7.4, containing 10 % glycerol and 0.05 % Triton X-100. The adsorbent pellets were then boiled for 5 min with 100 μl of electrophoresis sample buffer containing 100 mM-DTT, centrifuged, and the supernatant taken for electrophoresis on SDS/polyacrylamide gels (7.5 % polyacrylamide). Gels were dried and autoradiographed as described (O'Brien *et al.*, 1987).

Immunoaffinity purification of insulin receptor

Human placental microsomes or rat liver membranes were solubilized as described above (final volume approx. 50 ml for one placenta or 100 g of rat liver). The extracts were incubated with CT-1 immuno-adsorbent, using 1 mg of cellulose/ml of extract for 2 h at 4 °C, or 20 mg of Sepharose/ml of extract for 5 h at 4 °C.

Cellulose adsorbent was collected by centrifugation at 2500 *g* for 10 min, and washed four times with 50 mM-Hepes, pH 7.4, containing 10 % glycerol, 1 M-NaCl, 0.05 % Triton X-100, and once in the same buffer without NaCl, all at 4 °C. Receptor was then eluted by incubating with peptide solution (normally 100 $\mu\text{g}/\text{ml}$) in a volume of buffer (50 mM-Hepes, pH 7.4, con-

taining 10 % glycerol and 0.05 % Triton X-100), equivalent to one-third of the volume of solubilized membranes taken initially. After incubation at 4 °C or room temperature as indicated, the adsorbent was pelleted and the supernatant taken and filtered through a 0.22 μl Millex GV filter to remove adsorbent fines.

Sepharose adsorbent was transferred to a suitable column after receptor binding, and washed at 4 °C with 10 column vol. of buffer (50 mM-Hepes, pH 7.4, 100 mM-NaCl, 0.05 % Triton X-100, together with proteinase inhibitors as for solubilization), at a flow rate of 1 column vol./h. Receptor was eluted at 4 °C with 5 vol. of the same buffer containing peptide (200 $\mu\text{g}/\text{ml}$), at a flow rate of 0.33 column vol./h.

Eluted receptor was concentrated where necessary on an Amicon ultrafiltration unit, using a Diaflo PM-30 membrane, or by binding to and elution from a 0.2 ml column of wheat germ agglutinin-agarose (Fujita-Yamaguchi *et al.*, 1983). Recovery of receptor was monitored by measuring ^{125}I -insulin binding in a PEG precipitation assay (Baron & Sonksen, 1982). The purified receptor was examined by SDS/PAGE. Samples were precipitated with 10 % trichloroacetic acid, centrifuged, and the precipitate extracted once with acetone before addition of electrophoresis sample buffer containing 100 mM-DTT. Samples were analysed on SDS/7.5 % polyacrylamide gels, which were stained first with Coomassie Blue and then destained before proteins were finally visualized by silver staining (Ansorge, 1982).

Construction and screening of a peptide library

The random peptide library was constructed by cloning oligonucleotides containing 23 random bases into pCL627, which is a variation of pEX627 (Stanley & Herz, 1987) having a unique *Hind*III site in the cloning linker. Standard techniques were carried out as described in Sambrook *et al.* (1989). The oligonucleotide 5'-CGGGT(N)₂₃GAAGCTTC-3' (100 pmol) was dissolved in 10 μl of high-salt restriction enzyme buffer, heated at 70 °C for 3 min, and then cooled to room temperature to allow annealing of the 8 bp self-complementary 3' ends. Extension by the Klenow fragment of *Escherichia coli* DNA polymerase resulted in a double-stranded dimer of the oligonucleotide with a central *Hind*III site. This dimer was purified on a Qiaagen tip, precipitated with ethanol and then ligated into the *Sma*I site of pCL627 using 10 units of T4 DNA ligase in a volume of 20 μl overnight at 20 °C. Ligase was denatured by heating to 70 °C for 3 min, the salt concentration was then adjusted, and the *Hind*III sites in the dimer and the vector polylinker were cut. After a further DNA-purification step, the excised *Hind*III fragments were removed on a 1 ml Sephacryl S400 spun-column run in 1 mM-Tris/HCl/0.1 mM-EDTA, pH 8. This leaves a single copy of the oligonucleotide sequence in the linearized vector. Recircularization via the *Hind*III sites was performed in a 30 μl volume using 5 units of T4 DNA ligase at 15 °C for 1 h. Vector molecules without insert were linearized again by cutting with 8 units of *Sal*I for 30 min. (The *Sal*I site in the vector falls between the *Sma*I and *Hind*III sites and is therefore lost in all constructs containing an oligonucleotide in the correct position.) The DNA was subjected to phenol/chloroform extraction and ethanol precipitation, taken up in 20 μl of water and micro-dialysed on a Millipore Vs filter. This DNA was then used to transfect freshly prepared pop2136 cells by electroporation. The yield was a library of more than 10⁷ clones, from which 22 clones were picked at random and sequenced. All of these contained a random sequence in the expected position. One clone had a 7 bp deletion which could have been caused by a *Hind*III site in the random sequence.

Colony blots of the expressed random library were screened as previously described (Stanley, 1988; Prigent *et al.*, 1990). Positive clones were colony purified and the vector inserts sequenced as

described by Tsang & Bentley (1988), using the primer sequence 5'-GAATTATTTTGGATGGCGTTAACTCGGCG-3', [32 P]-dCTP as label, and a minus-dCTP, minus-dGTP labelling mix. This results in the addition of eight nucleotides including two radiolabelled C residues on every extended primer before the termination reaction, allowing the sequence to be determined close to the primer sequence. The amino acid sequence encoded by the oligonucleotide at the C-terminus of the β -galactosidase fusion protein is PG(X)₈KLAD or, with a single-base frame shift, PG(X)₈SLLD.

RESULTS

Production and characterization of antibody CT-1

Mice and rabbits were injected with KLH-conjugated peptide corresponding to the C-terminal 15 amino acids of the human insulin receptor, and sera were screened for production of anti-receptor antibodies by testing for binding of receptor- 125 I-insulin complexes. The use of this assay, rather than a screening method based on peptide binding, ensured that only antibodies reacting with native insulin receptor were detected. Each of four rabbits but only two of ten mice developed significant serum antibody titres after five injections (binding > 20% of receptor-insulin complex at 10^{-3} serum dilution). The two positive mice were given a further injection of peptide conjugate together with purified insulin receptor before fusions were carried out. The products of each fusion were distributed on four microtitre trays and hybridoma growth was obtained in more than 90% of the wells. Two positive wells were detected in the receptor-binding assay but only one of these cell lines was successfully cloned. The corresponding antibody, coded CT-1, was identified as an IgG1 by dot-blot assay with antisera specific for mouse immunoglobulin isotypes.

It was confirmed that antibody CT-1 recognized the C-terminal receptor sequence corresponding to the peptide used as primary immunogen. Thus the free synthetic peptide (15-mer, KKNRILTLPRSNPS) competitively inhibited the interaction of CT-1 with receptor in the standard screening assay, with half-maximal inhibition at 25 nM-peptide (Fig. 1). A shorter peptide (11-mer, RILTLPRSNPS) was approximately one-third as potent on a molar basis, but a still shorter sequence (7-mer, LPRSNPS) was without effect up to the highest concentration tested (13 μ M). The C-terminal peptide (15-mer) did not affect binding of a previously characterized monoclonal antibody 18-40, which recognizes a known epitope (Prigent *et al.*, 1990) on the extracellular portion of the β -subunit (results not shown).

Antibody CT-1 reacted equally with rat and human insulin receptors. When solubilized rat liver plasma membranes were used in place of human placental membranes in the standard screening assay, the antibody displayed identical titres for binding receptor from both sources (Fig. 2). The mouse insulin receptor also reacted with antibody CT-1 (results not shown). This is consistent with the known sequences of rodent and human receptors which are identical in the C-terminal 15 amino acids apart from the presence of valine (rat, mouse) rather than isoleucine (human) ten residues from the terminus (Ebina *et al.*, 1985; Flores-Riveros *et al.*, 1989; Goldstein & Dudley, 1990). Antibody CT-1 was approximately one-fifth as effective on a concentration basis as antibody 83-14 (Fig. 2). This latter antibody reacts with the human but not the rat receptor α -subunit (Soos *et al.*, 1986; Prigent *et al.*, 1990) and is the most efficient of previously available anti-receptor monoclonal antibodies for immunoprecipitation.

Antibody CT-1 did not react with the human type-1 IGF receptor, but did react with insulin-IGF receptor hybrids. When solubilized type-1 receptor from transfected IGF-1R/3T3 cells

was employed in a screening assay with 125 I-IGF-I, no reaction of CT-1 was detected (results not shown), although there was good reaction with the IGF-I receptor-specific antibodies 16-13 and α -IR-3 (Soos *et al.*, 1990). However, significant binding of receptor- 125 I-IGF-I complexes in solubilized human placental

Fig. 1. Inhibition of receptor binding to antibody CT-1 by free peptide

Partially purified human insulin receptors were preincubated with 125 I-insulin for 16 h at 4 °C such that 15% of the insulin was bound as determined by PEG precipitation. Samples of the receptor- 125 I-insulin complex were incubated with antibody CT-1 (0.2 μ g/ml) and synthetic peptide (concentrations as indicated) in 200 μ l of binding assay buffer for 2 h at 4 °C. Sheep anti-mouse IgG immunoadsorbent (0.5 mg of cellulose) was then added for 30 min. The immunoadsorbent was collected by centrifugation and washed three times before determination of radioactivity. Maximum bindable receptor- 125 I-insulin complex with excess CT-1 (2 μ g/ml) was 2800 c.p.m., and non-specific binding in the absence of CT-1 was 110 c.p.m. Each point is the mean of duplicate determinations. The peptides used were: 15-mer KKNRILTLPRSNPS (●); 11-mer RILTLPRSNPS (○); 7-mer LPRSNPS (□).

Fig. 2. Reaction of antibody CT-1 with rat insulin receptors

Solubilized membranes from human placenta or rat liver were preincubated with 125 I-insulin for 16 h at 4 °C such that 15% of the insulin was bound as determined by PEG precipitation. Samples of the receptor- 125 I-insulin complex were incubated with antibody CT-1 (▲, human placenta; △, rat liver) or 83-14 (●, human placenta; ○, rat liver) at the indicated concentrations in 200 μ l of binding assay buffer for 1 h at 4 °C. Sheep anti-mouse IgG immunoadsorbent (0.5 mg of cellulose) was then added for 30 min. The immunoadsorbent was collected by centrifugation and washed three times before determination of antibody-bound radioactivity. Total receptor-bound radioactivity was determined in parallel samples by PEG precipitation. Immunoprecipitable radioactivity is expressed as percentage of PEG-precipitable radioactivity. Values are means of duplicate incubations.

membranes by CT-1 was observed (results not shown), consistent with the presence of insulin-IGF-I hybrid receptors in placenta as previously demonstrated with other insulin receptor antibodies (Soos & Siddle, 1989).

It has been reported that some antibodies for epitopes close to the C-terminus of the insulin receptor β -subunit show differential reactivity dependent on conformational changes induced by autophosphorylation of the receptor and/or insulin binding (Baron *et al.*, 1990). It was therefore confirmed that CT-1 reacted with autophosphorylated and unoccupied receptors as well as the receptor-insulin complex used in the screening assay. Thus antibody CT-1 precipitated autophosphorylated receptor as efficiently as did other monoclonal antibodies which recognize epitopes in the extracellular portion of the receptor (Fig. 3). The antibody also removed approx. 80% of the unoccupied non-phosphorylated receptors from solubilized placental membranes, as detected by insulin-binding assays performed on the original and depleted extracts (results not shown). The residual binding activity was not removed by a second depletion with CT-1, but was removed by the α -subunit-reactive monoclonal antibody 83-14. Similarly, the maximum amount of receptor- ^{125}I -insulin complexes bound by CT-1 in the standard screening assay was only 80–90% of that precipitable with PEG, whereas, with 83-14, the value was more than 95%. The fraction of receptors that was unreactive with CT-1 whether or not insulin was bound (10–20% in different preparations from placenta or CHO.T cells) may in part reflect selective loss of the CT-1 epitope by limited proteolysis. It has been shown that the C-terminal segment of the receptor is particularly susceptible to proteolysis (Grunfeld *et al.*, 1987; Shoelson *et al.*, 1989).

Antibody CT-1 reacted specifically with the denatured insulin receptor in whole-cell extracts. When nitrocellulose blots of proteins from CHO.T cells (Ellis *et al.*, 1986) were probed with CT-1, two reactive proteins were identified (Fig. 4). These corresponded in electrophoretic mobility to the β -subunit of the insulin receptor (95 kDa) and the uncleaved proreceptor (approx. 200 kDa) which is overexpressed in these cells. The same two components reacted with each of the rabbit anti-peptide sera coded Ros-1, -2, -3 and -4. Antisera to whole receptor additionally reacted with α -subunit (135 kDa). A component of approx. 85 kDa, which may have been partially proteolysed β -subunit lacking an intact C-terminus, was detected when blots of CHO.T extract were probed with a polyclonal anti-receptor serum but not with CT-1 or Ros-1.

The epitope for CT-1 is obviously remote from the binding site for insulin, which is in the extracellular α -subunit of the receptor (Waugh *et al.*, 1989; Andersen *et al.*, 1990; Gustafson & Rutter, 1990). It was therefore somewhat surprising that, when solubilized receptor was preincubated with antibody CT-1, binding of ^{125}I -insulin was stimulated 1.6-fold (Fig. 5). The concentration-dependence of this effect closely paralleled that for antibody binding to the receptor. Allowing for the fact that only approx. 80% of the receptor reacted with CT-1, the true stimulation of insulin binding in the reactive fraction was approx. 2-fold. As would be expected, the antibody was without effect on binding of insulin to intact cells (IM-9 lymphocytes or CHO.T fibroblasts; results not shown).

Antibody CT-1 increased the basal rate of receptor autophosphorylation approximately 1.7-fold but did not affect insulin-stimulated autophosphorylation (Fig. 6). Unlike the stimulation of autophosphorylation induced by antibodies reacting with the extracellular portion of the receptor (O'Brien *et al.*, 1987), the stimulation by CT-1 did not show a narrow antibody concentration-dependence. This effect of CT-1 was specific to the insulin receptor and there was no effect on the phosphorylation of other proteins within the crude receptor

Fig. 3. Precipitation of autophosphorylated receptor by antibody CT-1

Solubilized placental membranes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, without (lane A) or with (lanes B–F) the addition of $0.1\text{ }\mu\text{M}$ -insulin. Equal samples were taken for loading on to SDS/polyacrylamide gels either directly (lanes A and B) or after immunoprecipitation with mouse monoclonal antibodies (lanes C–F) as described in the Experimental section. The dried gels were subjected to autoradiography. The antibodies used were anti-(human insulin receptor), 83-7 (lane C) and anti-(human insulin receptor), 25-49 (lane D) (Soos *et al.*, 1986), anti-(human thyrotropin), D70 as a control IgG1 antibody of irrelevant specificity (lane E) and anti-peptide CT-1 (lane F).

Fig. 4. Immunoblot of CHO.T cell lysate with anti-receptor antibodies

A lysate of CHO.T cells was electrophoresed on a $20\text{ cm} \times 20\text{ cm}$ SDS/polyacrylamide gel (7.5% polyacrylamide), and blotted on to nitrocellulose which was then cut into 1 cm strips. Each strip contained extract from approx. 5×10^5 cells and therefore approx. 1 pmol ($0.5\text{ }\mu\text{g}$) of insulin receptors. The nitrocellulose strips were incubated with anti-receptor antibody (1:100 dilution of mouse ascites fluid or rabbit serum) and then with appropriate peroxidase-conjugated second antibody as described in the Experimental section. Lane A, non-immune control (mouse monoclonal IgG1 of irrelevant specificity); lane B, mouse monoclonal antibody CT-1; lane C, mouse monoclonal anti-(human insulin receptor), 83-14 (Soos *et al.*, 1986); lane D, rabbit anti-(C-terminal peptide), Ros 2; lane E, rabbit serum Rab 1 anti-(human insulin receptor) (Soos *et al.*, 1986).

preparation except for a decrease in phosphorylation of a protein of approx. 60 kDa. The phosphorylation of this unidentified protein was not insulin-dependent, nor did it increase with time

Fig. 5. Effect of antibody CT-1 on insulin binding

Insulin receptor solubilized from human placental membranes was preincubated with antibody CT-1 at the indicated concentrations in 200 μ l of binding assay buffer for 6 h at 4 °C before addition of 125 I-insulin (50 μ l, 30 000 c.p.m.) for a further 16 h. Receptor- 125 I-insulin complexes were then precipitated with PEG (●) or with sheep anti-mouse IgG immunoabsorbent (○) as described in the Experimental section and the legend to Fig. 2. Results shown are the means of duplicate determinations, corrected for non-specific precipitation in the absence of receptor (200 c.p.m. and 40 c.p.m., for PEG and immunoabsorbent precipitation respectively).

Fig. 6. Effect of antibody CT-1 on receptor autophosphorylation

Solubilized placental membranes were preincubated for 15 min at 22 °C with partially purified CT-1 (20 μ g/ml), in the absence or presence of insulin (0.1 μ M). Reaction mixtures were then placed on ice for 10 min before initiation of autophosphorylation by addition of [γ - 32 P]ATP (O'Brien *et al.*, 1987). The reaction was allowed to continue at 0 °C for the indicated times and terminated by addition of electrophoresis sample buffer. Samples were electrophoresed on SDS/polyacrylamide gels (10% polyacrylamide) which were stained, dried and subjected to autoradiography. (a) Basal (no insulin) phosphorylation; (b) insulin-stimulated phosphorylation. The intensity of phosphorylation of the 95 kDa band corresponding to the insulin receptor β -subunit was quantified by densitometric scanning of the autoradiograms using a Joyce-Loebl Chromoscan 3. The mean ratio of phosphorylation with or without CT-1 was 1.7-fold (basal) and 1.0-fold (insulin-stimulated).

between 5 and 60 min of incubation. It is assumed that the protein is a component of the crude placental preparation which is unrelated to the insulin receptor and which is phosphorylated

Fig. 7. Detailed analysis of the CT-1 epitope

Sequences reactive with antibody CT-1 were identified by screening a library of random peptides expressed in *E. coli*. To identify positive clones, colony blots were incubated with antibody CT-1 (1/100 dilution of ascites fluid) and then with alkaline phosphatase-conjugated second antibody, and finally developed with a Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate system as described (Stanley, 1988; Prigent *et al.*, 1990). Positive colonies were clearly distinguished by eye, and the strength of reaction was assessed objectively by scanning on an Omnimedia 6cx scanner at 300 dpi resolution, with 256 gray scales. For scanning, a small frame (approx. 1 mm²) was placed with the diagonal on the rim of the stained colony and quantified using the program IMAGE. Similar scans of Western blots were linear with the amount loaded for a given protein, within the range of intensity involved. Sequences of the various CT-1-reactive clones, numbered 1–6, are grouped according to reaction intensity. Amino acid identities compared with the human insulin receptor sequence are shown in bold, and regions of identity or conservative substitution within each group are boxed. A continuing sequence of *m* amino acids is indicated by (N)_{*m*}, and stop codons by *. The reaction intensity of each clone is the mean ± S.E.M. density (arbitrary units) from four determinations. The mean density for negative colonies was 5.4 ± 1.4 units. For comparison, the C-terminal sequences of the human insulin receptor (Ullrich *et al.*, 1985; Ebina *et al.*, 1985), the rat/mouse insulin receptor (Flores-Riveros *et al.*, 1989; Goldstein & Dudley, 1990) and the human IGF-I receptor (Ullrich *et al.*, 1986) are also shown.

by an unknown contaminating kinase. The significance of the inhibitory effect of partially purified CT-1 is unclear, but most likely it results from impurities in the antibody preparation.

Analysis of CT-1 epitope by screening a library of random peptides

The reactivity of CT-1 with peptides of different lengths (Fig. 1) suggested that the full 15-amino-acid sequence of the immunogen was not essential for high-affinity recognition by the antibody, but the C-terminal 7-amino-acid sequence was not sufficient. To analyse the CT-1 epitope in more detail, a library of random hexapeptide sequences was screened for reaction with the antibody. The peptide library was generated by synthesis of oligonucleotides containing 23 random bases which were ligated in the vector pCL627. After transformation of *E. coli* with the resulting plasmid library, peptides were expressed as fusion proteins with β -galactosidase and a colony blot was probed with antibody CT-1. The peptide sequences of reactive colonies were identified by sequencing the oligonucleotide inserts in purified plasmids.

From a screen of 900 000 colonies, seven positives were identified with six different sequences (Fig. 7), confirming that the library does indeed contain a large number of independent

Fig. 8. Reaction of CT-1 with threonine-phosphorylated peptide

A competitive e.l.i.s.a. was used to compare the reactivity of different peptides with antibody CT-1. The wells of a microtitre plate were coated with BSA-conjugated 15-mer peptide, 5 $\mu\text{g}/\text{ml}$ in 100 μl of TBS (50 mM-Tris/HCl, pH 7.4, 150 mM-NaCl), for 2 h at room temperature. The wells were then washed three times with TBS/0.1% Tween 20, blocked by incubation overnight with BSA, 10 mg/ml in 200 μl TBS, and again washed three times. For the assay itself, peptides at the concentrations shown were incubated together with antibody CT-1 (10 nM), in 100 μl of TBS per well, for 1 h at room temperature. The wells were then washed and incubated for a further 1 h with 100 μl of a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). After further washing, bound antibody was quantified by using 10 mM-*p*-nitrophenyl phosphate as substrate and measuring absorbance at 405 nm on a microtitre plate reader after 1 h of reaction. Maximum absorbance (in the absence of peptide) was 1.10 and background absorbance (in the absence of CT-1) was 0.08. Results are expressed as percentage of maximum binding after subtraction of background. The peptides used were: 15-mer KKNRILTLPRSNPS (●); 11-mer RILTLPRSNPS (○); phospho-11-mer RILT(PO₃)LPRSNPS (▲).

Fig. 9. Dissociation of CT-1-receptor complex

Rat liver insulin receptor (2 ml of solubilized membranes) was incubated with 2 mg of cellulose-CT-1 immunoadsorbent for 2 h at 4 °C. The immunoadsorbent was pelleted, washed and resuspended in 2 ml of buffer containing 15-mer peptide KKNRILTLPRSNPS (30 $\mu\text{g}/\text{ml}$). After incubation for various times at 4 °C (○) or 22 °C (●), samples were removed and centrifuged, and serial dilutions of the supernatant were taken for insulin-binding assay. The dilution which bound 5% of added ¹²⁵I-insulin was estimated as a measure of relative receptor concentration in different samples. The amount of receptor initially bound was calculated as the difference between the concentration in the rat liver extract and the supernatant after incubation with immunoadsorbent. Dissociated receptor was expressed as percentage of that initially bound. Each point is the mean of duplicate incubations.

sequences. Surprisingly, only two out of these six different positive sequences had the full segment of 23 random bases, compared with 21 out of 22 clones in a random selection. This indicates a very selective bias introduced by the specificity of the CT-1 antibody. Furthermore, the two clones with the expected size insert (numbers 4 and 6 in Fig. 7) only scored medium or weak binding of the CT-1 antibody. All of the positive clones showed a span of sequence which was closely related to the sequence of the insulin receptor peptide immunogen. The strongest reactions were obtained with peptides having seven or eight sequence identities with the insulin receptor within an internal span of nine amino acids [(G)RVL(T)LPRSL]. In both these clones a reading-frame shift gave rise to a serine from the vector-derived sequence immediately after the random sequence. Thus, in effect, the antibody selects out a small population of nine-residue random sequences from a library predominantly containing random octapeptides. This implies that the epitope is actually quite long, since 8-mer sequences ending in a serine were not detected in the screening. An analysis of the sequences of clones giving weaker reactions is also informative. Particularly interesting is the weakest positive, clone 6, which contains six identities compared with seven or eight for the strongly reacting clones. Presumably the missing amino acids (LXXPX) are very important for antibody binding, and the substitution of arginine for leucine and lysine for proline within this motif may have a particularly adverse effect on binding. In confirmation of this hypothesis, clones 4 and 5, which each contain only two perfect matches with the insulin receptor sequence, but which show relative conservation of the LXXPR motif, gave a stronger reaction than clone 6 which is missing this motif.

The results of the random peptide screening (Fig. 7) were in agreement with the limited epitope analysis using synthetic peptides (Fig. 1) in confirming that the three residues at the *N*-terminus of the 15-mer peptide immunogen were not important for binding of CT-1. The analysis of random peptides further showed that the three residues at the *C*-terminus of the immunogen and the receptor were also not required for antibody binding. The sequence LAD (or LID), which appears at the *C*-terminus of several of the positive random peptides, derives from the common region of oligonucleotides used in the library construction, and would be present in the majority of clones in the library. This sequence presumably does not contribute to binding as it must be present in many negative clones and is absent from other reactive peptides and from the receptor itself.

It appeared from the epitope analysis that Thr-1348, a known phosphorylation site of the human receptor (Lewis *et al.*, 1990), was not important for CT-1 binding, as a strong or medium reaction was obtained even when this was replaced non-conservatively by histidine (clone 1) or cysteine (clone 5). However, the affinity of CT-1 for threonine-phosphorylated 11-mer peptide was approx. 100-fold less than for the corresponding non-phosphorylated peptide when compared in an e.l.i.s.a. (Fig. 8). It is therefore likely that the antibody will react relatively poorly with receptor phosphorylated at Thr-1348. This may account in part for the incomplete precipitation of receptor by CT-1 compared with other antibodies, although at least under basal conditions the stoichiometry of phosphorylation at this site appears low (Lewis *et al.*, 1990), and the chances that both β -subunits within a native heterotetrameric receptor are simultaneously phosphorylated must therefore be small.

Immunoaffinity purification of receptor using antibody CT-1

A problem in many immunoaffinity-purification procedures is that the conditions necessary for elution of antigen from antibody may not be compatible with retention of normal function in the recovered antigen. In the present instance, an antibody was

Table 1. Immunoaffinity purification of insulin receptors

Solubilized human placental membranes were incubated with cellulose-CT-1 for 2 h at 4 °C (A, B), and solubilized rat liver membranes were incubated with Sepharose-CT-1 for 5 h at 4 °C (C), as described in the Experimental section. Immunoadsorbents were washed extensively before elution of receptor with a solution of peptide (15-mer), batchwise for 2 h at 4 °C (A) or 2 h at 22 °C (B), or in a column overnight at 4 °C (C), again as described in the Experimental section. Insulin-binding assays were carried out on the solubilized membranes, the supernatant after incubation with immunoadsorbent, and the eluted material, in order to calculate the fraction of receptor bound to immunoadsorbent and recovered in the final eluate. Results are means $\pm$ S.E.M. of the number of independent experiments shown, involving 2–5 ml (A, B) or 50–90 ml (C) of solubilized membranes.

Source of receptor (adsorbent/elution)	Receptor bound to immunoadsorbent (%)	Receptor recovered in eluate (%)
A Human placental membranes (Cellulose-CT-1, elution 2 h at 4 °C) (n = 8)	76 $\pm$ 2	31 $\pm$ 2
B Human placental membranes (Cellulose-CT-1, elution 2 h at 22 °C) (n = 7)	74 $\pm$ 2	68 $\pm$ 5
C Rat liver membranes (Sepharose-CT-1, elution 16 h at 4 °C) (n = 4)	80 $\pm$ 4	54 $\pm$ 4

Fig. 10. Immunoaffinity purification of human and rat insulin receptors

Human placental insulin receptor (2 ml of solubilized membranes) was incubated with 2 mg of cellulose-CT-1 immunoadsorbent for 2 h at 4 °C. The immunoadsorbent was pelleted, washed and resuspended in 0.6 ml of buffer (50 mM-Hepes, pH 7.4, 10 % glycerol, 0.05 % Triton X-100) containing 15-mer peptide KKNGRILTLPRSNPS (100 μ g/ml). After 2 h at 22 °C, the reaction mixture was centrifuged and the supernatant taken and filtered to remove adsorbent fines. Half of the eluate was precipitated with trichloroacetic acid for analysis by SDS/PAGE, and the remainder was used to estimate recovery of receptor by insulin-binding assay. The photograph shows a silver-stained 7.5 % polyacrylamide gel. Lane A, 1 μ l of solubilized placental membranes; lane B, purified receptor equivalent to 1 ml of solubilized placental membranes. The overall yield of receptor as determined by insulin-binding assay was 70 %.

available for which the peptide epitope was precisely known and for which it had been shown that free peptide could competitively inhibit binding of the native protein antigen. An immunoaffinity purification scheme was therefore developed in which insulin receptor was bound to immobilized CT-1 and recovered under very mild conditions by incubation in the presence of excess free peptide.

Membrane preparations from 100 g of human placenta or rat liver, when solubilized in 50 ml of buffer containing 2 % Triton X-100, yielded similar amounts of receptor as determined by 125 I-insulin binding assay. Immunoadsorbents for use in receptor purification were prepared by coupling purified antibody CT-1 to finely divided aminocellulose (200 mg of antibody per g of cellulose) or to Sepharose beads (10 mg of antibody per g of Sepharose). Solubilized receptor was incubated with immobilized antibody, batchwise with end-over-end mixing, and the immunoadsorbents were then pelleted by centrifugation and washed with buffer at 4 °C. Receptor concentrations in the original preparation and in supernatants after incubation with immunoadsorbent were determined by 125 I-insulin-binding assay. To bind 80 % of the insulin receptors during a 2 h incubation at 4 °C it was necessary to use approx. 200 μ g of immobilized CT-1 per ml of solubilized membranes, with either cellulose or Sepharose adsorbent. Longer incubation times decreased somewhat the amount of antibody required, but under all conditions it was necessary to use a large excess of antibody over receptor (probably at least 10-fold). This presumably reflects the unavailability of a large fraction of combining sites after immobilization of the antibody, due to steric hindrance from the solid-phase matrix. As mentioned previously, 10–20 % of the receptors in all preparations failed to bind to CT-1 apparently because of loss of the epitope through limited proteolysis.

The rate of dissociation of receptor from immobilized antibody was investigated by incubating the cellulose-CT-1–receptor complex with free peptide (Fig. 9). The concentration of peptide was calculated to be approximately a 5-fold molar excess over the total antibody-combining sites and sufficient completely to inhibit rebinding of dissociated receptor (compare with Fig. 1). Half-times for receptor dissociation were estimated to be 40 min at 22 °C and 4 h at 4 °C, for both human and rat receptors. The recovery of receptor was therefore strongly dependent on elution time and temperature, but overall recoveries of 60–70 % were readily achievable by eluting for 16 h at 4 °C or 2 h at 22 °C.

Two alternative purification protocols were devised either using cellulose adsorbent in a batch procedure or Sepharose adsorbent in a column (see the Experimental section). Cellulose adsorbents lack the flow properties necessary for column use, and Sepharose adsorbents occupy too great a volume for convenient batch use. Both procedures gave similar yields of receptor in terms of insulin-binding activity (Table 1). When analysed by SDS/PAGE, the purified receptor exhibited only two major bands corresponding to α - and β -subunits (Fig. 10), and these accounted for 70 % of the recovered protein as estimated by densitometric scanning of the stained gel. The yield of tyrosine kinase activity could not be determined precisely because of the difficulty of specifically measuring the insulin receptor kinase in crude solubilized membranes. However, the purified receptor retained at least 30 % of insulin-stimulated autophosphorylation compared with crude preparations, when normalized for recovery of insulin binding (results not shown).

DISCUSSION

We describe here the properties of a site-specific monoclonal anti-peptide antibody, CT-1, which is the first such antibody to be produced for the insulin receptor. The antibody, which reacts

with the C-terminal sequence of the receptor β -subunit, was used as a model to validate a general method of epitope mapping and to establish an immunoaffinity procedure for receptor purification. The same peptide antigen has previously been used to produce polyclonal antisera which discriminate between the insulin receptor and type-1 IGF receptor (Herrera *et al.*, 1986) or between intact and proteolysed forms of the insulin receptor (Grunfeld *et al.*, 1987). Antibody CT-1 reacted with rat and human insulin receptors, in both native and denatured states, and in both unphosphorylated and autophosphorylated forms. However, phosphorylation of a threonine residue corresponding to the known insulin receptor phosphorylation site Thr-1348 (Lewis *et al.*, 1990) considerably decreased the affinity of antibody binding to synthetic peptide. The relative affinity of the antibody for free unphosphorylated peptide and native receptor was not determined, but CT-1 was similar to other monoclonal anti-receptor antibodies (Soos *et al.*, 1986) in titre and capacity to immunoprecipitate receptors. The cross-reaction of CT-1 with rodent receptors would be expected from the near-identity of amino acid sequence with the human receptor in this region. Other monoclonal antibodies for four different epitopes in the intracellular portion of the receptor similarly reacted with both human and rat receptors (Morgan *et al.*, 1986). However, the epitopes for these antibodies were 'conformational' and the antibodies did not recognize denatured receptor (Morgan & Roth, 1986). None of the monoclonal antibodies for the extracellular portion of the human receptor which have been described to date react with rat receptors, although there is a very high degree of sequence similarity between rat and human receptors in all domains (Morgan & Roth, 1986; Soos *et al.*, 1986).

Antibodies have been used to detect conformational changes in the insulin receptor associated with activation of the tyrosine kinase. Anti-peptide antibodies for a sequence including the sites of regulatory phosphorylation (tyrosines-1158, -1162, -1163) react with the phosphorylated but not the unphosphorylated receptor (Herrera & Rosen, 1986; Perlman *et al.*, 1989), whereas various monoclonal antibodies for the kinase domain show decreased reactivity with phosphorylated compared with unphosphorylated receptors (Morgan & Roth, 1986). Insulin binding alone does not induce such conformational changes in the regulatory region (Perlman *et al.*, 1989), although binding of ATP without accompanying phosphorylation may do so (Maddux & Goldfine, 1990). In contrast, it has been reported that conformational changes in the C-terminal region are induced by insulin binding, as detected by anti-peptide antibodies reacting between residues 1306 and 1338. However, in the present work we found that antibody CT-1 reacted well with free unphosphorylated receptor, receptor-insulin complexes and autophosphorylated receptor. Thus any conformational changes induced by insulin binding or autophosphorylation do not dramatically affect immunoreactivity of the extreme C-terminus (residues 1344–1355).

Surprisingly antibody CT-1 did influence both insulin binding and basal tyrosine kinase activity of solubilized receptors, although the epitope is remote from both the kinase active site and, especially, the insulin-binding site. These effects of antibody may be a consequence of conformational changes secondary to receptor aggregation or intramolecular cross-linking of β -subunits, as seen with antibodies reacting in the extracellular portion of the receptor (O'Brien *et al.*, 1987). Interestingly, it has been reported that insulin-binding affinity in some cell types is decreased by phorbol ester treatment (Grunberger & Gorden, 1982; Thomopoulos *et al.*, 1982). Phorbol esters, by activating protein kinase C, induce C-terminal phosphorylation of the insulin receptor at Thr-1348 (Koshio *et al.*, 1989; Lewis *et al.*,

1990). It is therefore possible that perturbation of the receptor C-terminus, by antibody binding or by phosphorylation, influences overall conformation so as to affect even the insulin-binding site. There was no detectable effect of CT-1 on insulin-stimulated receptor autophosphorylation, even though the antibody binds close to two major phosphorylation sites at Tyr-1328 and -1334 (Tornqvist *et al.*, 1987; Tavaré & Denton, 1988). It is possible that changes in phosphorylation at these sites might go undetected against the background of phosphorylation at other sites.

The value of antibodies as site-specific reagents for use in the purification, assay and functional analysis of proteins is greatly enhanced if their epitopes are known. Synthetic peptides have become widely used as antigens to produce antibodies of predetermined specificity (Lerner, 1984). In the case of the insulin receptor a variety of rabbit polyclonal antibodies of this type have been described although not all of these react with the corresponding sequences in intact native receptor (Herrera *et al.*, 1985; Herrera & Rosen, 1986; Perlman *et al.*, 1989; Pessina *et al.*, 1989; Baron *et al.*, 1989, 1990; Toyoshige *et al.*, 1989; Rosenzweig *et al.*, 1990). Moreover, as with all polyclonal antibodies, limited availability restricts the potential applications of these reagents. Various monoclonal antibodies have been produced using intact insulin receptor as immunogen (Roth *et al.*, 1982; Kull *et al.*, 1983; Morgan & Roth, 1986; Soos *et al.*, 1986; Forsayeth *et al.*, 1987). Peptide epitopes have been mapped subsequently for some of these, although most react with 'conformational' determinants and do not recognize denatured receptor or short peptides (Morgan & Roth, 1986; Prigent *et al.*, 1990). We have previously screened a library of receptor peptide sequences expressed from fragments of cDNA in order to identify epitopes for monoclonal and polyclonal antibodies. We describe here a more general approach to epitope mapping using a library of random peptide sequences expressed from synthetic oligonucleotides. This technique provides an alternative to methods based on chemical synthesis of peptides (Houghten, 1985; Geysen *et al.*, 1987) for the identification of epitopes and mimotopes. Using a pEX family expression vector also had the advantage that large quantities of fusion protein were expressed, allowing antibody-reactive clones to be detected easily. The library of 10^7 colonies generated as a plasmid-expression system was obviously not fully representative of the theoretical number of 2.6×10^{10} different octapeptide sequences. Nevertheless this approach allows the screening of a vastly greater number of sequences than could conveniently be generated by chemical synthesis, and lends itself to the analysis of epitopes for which there is no prior sequence information. Furthermore, although there might be only 1 in 2.6×10^{10} perfect matches, strong positives were observed for peptides having conservative amino acid replacements (for example K for R or V for L). Calculating on the basis of nine groups of similar amino acids, the size of library required to give representative octapeptide sequences would be only 4.3×10^7 . As seen here, weak positives with many mismatches also give informative results so long as they can be unambiguously aligned. Thus libraries of manageable size are appropriate for this type of screening. The advantages of increasing the size of the random peptide segment are that larger epitopes can be detected and all antibody binding is stronger. Indeed, one of the surprising results of the present analysis was that the epitope of CT-1 spans as many as nine amino acids. A library encoding a random 10-mer or even 12-mer might therefore be preferable.

In the present work, six different sequences reactive with antibody CT-1 were identified, which showed varying degrees of similarity to the predefined peptide antigen. Surprisingly, the epitope is internal within the peptide sequence, despite the

nearby C-terminus of the peptide antigen. This suggests that the CT-1 antibody and epitope will be useful for foreign epitope tagging of proteins expressed from engineered DNA constructs, as the sequence can in principle be introduced within as well as at the terminus of constructs. Screening of the random peptide library permitted not only unambiguous identification of the receptor epitope but also assessment of the relative importance of individual amino acids within the epitope, and identification of various peptide sequences which could in principle be used competitively to inhibit interaction of CT-1 with the receptor. It should be possible to extend this approach to antibodies which recognize 'conformational' epitopes and therefore fail to react with short peptides derived directly from the primary sequence of a protein antigen. In such cases it may nevertheless be possible to identify peptides within a random library which present an appropriate array of residues to act as 'mimotopes'. Such peptides could then be used competitively to inhibit interaction of antibody with protein antigen.

We made use of the binding competition between free peptide and protein antigen in an immunoaffinity purification of insulin receptors. Previously described techniques for purification of insulin receptors using specific monoclonal antibodies have required the use of potentially denaturing conditions (Roth & Morgan, 1985; O'Brien *et al.*, 1986; Roth *et al.*, 1986) or of insulin (Roth & Morgan, 1985) to elute receptor from immobilized antibody. In the present work native receptor was specifically recovered from immobilized antibody CT-1 by incubating under very mild conditions with an excess of free peptide related to the known epitope. In general, the efficiency of such elution will depend on the intrinsic rate of dissociation of the antibody-antigen complex (without deliberate perturbation by alteration of pH or addition of chaotropes), and the ability of free peptide to inhibit competitively reassociation of the receptor with the antibody. In this instance the half-time for dissociation of receptor from the anti-peptide antibody was very temperature-dependent but short enough even at 4 °C ($t_{1/2}$ of 4 h) to allow good recovery of receptor with an elution period of 6–16 h. In addition to allowing the purification of solubilized receptors, this approach could be adapted for immunoaffinity isolation (Baillyes *et al.*, 1987; Howell *et al.*, 1989) of receptor-containing membrane vesicles. Since the epitope for CT-1 mapped to an internal sequence, this antibody and epitope could be used for the purification of any protein (or organelle in the case of membrane proteins) for which an oligonucleotide encoding the CT-1 epitope can be cloned into an exposed portion of the molecule. Analogous procedures might be used more generally with any protein for which site-specific antibodies are available, provided that short peptides reacting with the antibody can be identified by screening a peptide library.

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