Vol. 155, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1988 Pages 59-65

# A SPECIFIC INSULIN RECEPTOR AND TYROSINE KINASE ACTIVITY IN THE MEMBRANES OF *NEUROSPORA CRASSA*

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Received June 30, 1988

Cells of the wall-less ("slime") strain of *Neurospora crassa* possess specific high affinity insulin binding sites on their cell surface. $^{125}$I-labeled bound insulin was not displaced from these cells by insulin-like growth factor II (IGF-II), and was only weakly displaced by IGF-I and proinsulin. Cross-linking of $^{125}$I-labeled insulin with *N. crassa* cells using disuccinimidyl suberate resulted in the labeling of a single band of ca. 67 kDa m.w. on a polyacrylamide gel. Two proteins of ca. 66 and 59 kDa m.w. were purified from detergent solubilized plasma membrane preparations by passage over an insulin-agarose affinity matrix. Antibodies against an autophosphorylation site on the human and Drosophila insulin receptors (anti P2) immunoprecipitated a single phosphoprotein of ca. 50 kDa m.w. from detergent solubilized plasma membranes, which possessed protein tyrosine kinase activity when histone H2 was used as substrate. 

Diverse molecules resembling specific mammalian peptide hormones in both immunological and biological properties have been found in *Drosophila* as well as in several lower eukaryotes and prokaryotes (reviewed in 1). We have previously shown that addition of mammalian insulin to a wall-less ("slime") strain of *Neurospora crassa* can exert extensive metabolic, morphological and growth effects (2,3). Metabolic effects include an increased rate of glycogen synthesis, attributable to a conversion of the D to the I form of the enzyme glycogen synthase, and an enhanced rate of overall glucose oxidation--all reminiscent of insulin's actions on mammalian glucose metabolism. Furthermore, high affinity insulin binding sites similar to those provided by mammalian insulin receptors were found by Scatchard analysis to be present on the surface of the *N. crassa* cells (2). In this paper we have further characterized the specificity of these insulin receptors, and have identified putative binding proteins, in

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0006-291X/88 $1.50

addition to another protein apparently related to a human insulin receptor tyrosine kinase domain.

## MATERIALS AND METHODS

Materials. All materials not specifically mentioned were obtained as described (2,3). Trans label ($^{35}$S-methionine and cysteine), $\gamma$ $^{32}$P-ATP, and $^{32}$P-phosphate (carrier free) were from ICN. $^{125}$I-mono-iodo (A14) insulin (receptor grade) was from NEN. Insulin-like growth factors, IGF-I and IGF-II were obtained from ICN. Porcine proinsulin was a gift from Ron Chance at Eli Lilly.

Cells. The "slime" or wall-less variant of *N. crassa* (FGSC 4761) was cultured in a rich defined medium as described previously (2). *In vivo* labeling of proteins was by the addition of either 200 $\mu$Ci/ml $^{35}$S-Trans-label or 200 $\mu$Ci/ml $^{32}$P-phosphoric acid to 18 hr cultures. Cells were collected by centrifugation after 3 hr labeling.

Insulin binding and cross-linking. Ligand specificity was assessed by a standard binding assay (4) after incubating cells overnight at 4° with $^{125}$I-insulin (0.1nM) and the indicated concentrations of competing ligands. Bound radioactivity was measured after centrifugation through a 1:1 mixture of dinonylphthalate and Dow 702 silicon oil, and 3 washes in binding buffer (4). For cross-linking studies the washed cells were washed a further 3 times in binding buffer minus BSA and cross-linked by the addition of disuccinimidyl suberate as described by Pilch and Czech (5).

Insulin affinity chromatography. Insulin-agarose was prepared using porcine insulin coupled to Affigel 10 (Biorad). The resin (25 ml packed volume) was washed with 400 ml of cold distilled water and then coupled for 3 hr at room temperature with 25 mg insulin in 0.1 M Hepes pH 7.6, 80 mM $CaCl_2$, and 6M Urea. Remaining active groups were blocked overnight by the addition of 2.0 ml of 1.0 M ethanolamine. The resin was washed extensively in 0.1 M Hepes pH 7.6, 6 M urea and then with 0.1 M Hepes pH 7.6 before equilibration in binding buffer (see below). Insulin coupling was monitored by the addition of trace amounts of $^{125}$I-insulin.

Cells were collected from overnight cultures by centrifugation at 5000 g for 10 min and homogenized in 5-10 vols of 0.25 M sucrose, 50 mM Hepes pH 7.6, 1 mM EDTA, 0.1 mM DTT, 0.1 mg/ml bacitracin, 40 $\mu$g/ml PMSF by 15 passes of a tight fitting pestle in a Dounce homogenizer. After centrifugation at 8000 g for 10 min the supernatant was collected and centrifuged for a further 60 min at 40000 g. The pellet, a crude plasma membrane fraction, was then homogenized in 10 vols of 2% v/v Triton X-100, 50 mM sodium acetate pH 6.3, 0.1 mg/ml bacitracin, 40 $\mu$g/ml PMSF, left on ice for 30 min and detergent-insoluble material removed by centrifugation (40000 g for 30 min). The concentration of detergent was lowered to 0.1% by brief dialysis and ion-exchange chromatography on DEAE-cellulose, the eluate dialyzed against 50 mM Hepes pH 7.4, 0.1% Triton X-100, 0.1 mg/ml bacitracin, 40 $\mu$g/ml PMSF and then finally adjusted to the following binding buffer: 50 mM Hepes pH 7.4, 150 mM NaCl, 5.0 mM KCl, 2.0 mM $MgSO_4$, 1.0 mM EDTA, 0.1% Triton X-100, 0.1% bacitracin, 40 $\mu$g/ml PMSF. The solubilized membrane preparation in this buffer was applied to the insulin-agarose matrix in the presence or absence of 10 $\mu$M insulin to determine specific binding. After extensive washing in binding buffer the matrix was used directly for kinase assays or resuspended directly in SDS-sample buffer (containing DTT in place of 2-mercaptoethanol).

Antibody preparation: Antibodies directed against a synthetic peptide comprising the human insulin receptor autophosphorylation site (RNIYETDYYRKGGKGLLPVR, corresponding to residues 1143-1162 in ref. 6; peptide P2), prepared as described by Herrera and Rosen (7), was originally a generous gift from Dr. Ora Rosen. To obtain later preparations, the peptide was synthesized, purified and coupled to keyhole limpet hemocyanin by the Protein Chemistry Laboratory, Center for Advanced

Biotechnology and Medicine, Piscataway, N.J. The conjugated peptide was used to raise polyclonal antibodies in rabbits using established procedures (8) and then purified on a column of the peptide coupled to Affigel 10.

Immunoprecipitation. A plasma membrane fraction was prepared by the method of Scarborough (9) and solubilized in 1.0% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.1 mg/ml bacitracin, 40 μg/ml PMSF. Insoluble material was removed by centrifugation at 13000 g for 30 min. Affinity purified anti-receptor antibody was added and the samples left on ice for 4 hr. Immune complexes were precipitated with Protein A-Sepharose (Pharmacia), washed 3 times in the above buffer, once in PBS and then analyzed by SDS-PAGE.

General: SDS-polyacrylamide electrophoresis was performed under reducing conditions as described (10). Alkaline treatment of gels was by the method of Cheng and Chan (11) with the modifications of Castellanos and Mazon (12). Fixed gels were treated with 1 M KOH at 55° C for 1 hr, refixed and then dried and subjected to autoradiography. Proteins separated on SDS-polyacrylamide gels were visualized by silver staining using the method of Switzer *et al.* (13).

## RESULTS

Insulin binding: In a previous paper (2) we have demonstrated specific, high affinity insulin binding to *N. crassa* cells. In order to further characterize this binding, the ligand specificity was assessed by a competition assay (Fig. 1). The specificity resembled that of the mammalian receptor in that $^{125}$I-porcine insulin binding was inhibited strongly by both porcine and bovine insulin, only weakly by porcine proinsulin and IGF-I and almost not at all by IGF-II. To identify the protein components involved in this binding activity, *N. crassa* cells were affinity labeled with $^{125}$I-insulin using the bifunctional cross-linking reagent disuccinimidyl suberate. A single protein of ca. 67 kDa was labeled by this procedure (Figure 2). Labeling was completely inhibited by the addition of 10 μM unlabeled insulin (data not shown). The involvement of a plasma membrane protein in this molecular weight range as an "insulin-receptor" was supported by the results of affinity chromatography on insulin-agarose. When a detergent solubilized plasma membrane enriched fraction was applied to this matrix, two *N. crassa* proteins, of ca. 66 and 59 kDa could be specifically bound (Figure 3). When assayed for kinase activity *in vitro*, the beads bearing specifically bound proteins could phosphorylate added histone 2B in an alkali resistant manner, suggestive of tyrosine phosphorylation (data not shown).

Antibody crossreactivity: A range of anti-insulin receptor antibodies were tested for cross-reactivity with *N. crassa* proteins without success. Cross-reactivity was observed, however, using an anti-peptide antibody, P2, raised to a sequence from the autophosphorylation site in the kinase domain of the human insulin receptor B subunit. This

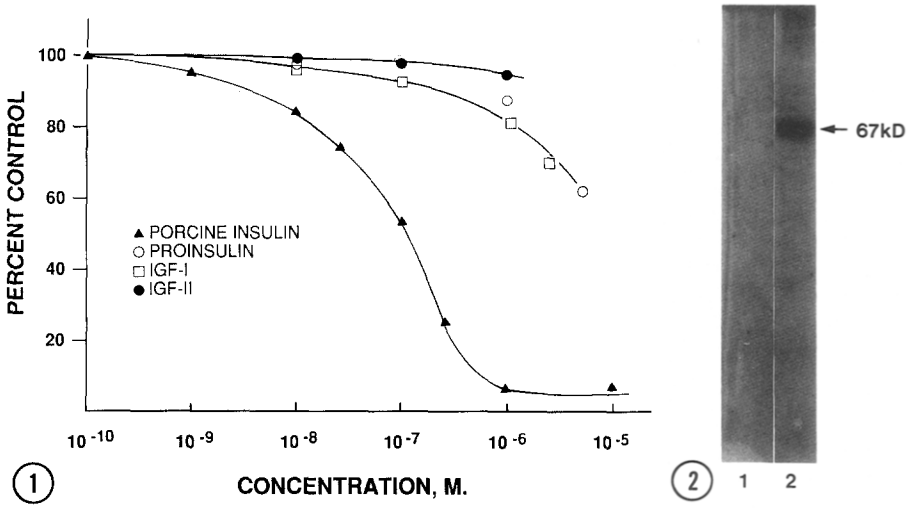


Figure 1. Displacement of $^{125}$I-insulin from intact *N. crassa* cells by: (▲), porcine insulin; (○), porcine proinsulin; (□), IGF-I; (●), IGF-II. Cells were incubated in the presence of 0.1nM $^{125}$I-insulin and the concentration of competing ligands shown. Cells were then washed, and specifically bound counts expressed as a percentage of control incubated in the absence of competing ligand.

Figure 2. Cross-linking of $^{125}$I-insulin to intact *N. crassa* cells. After washing, cells were incubated for 15 min on ice without (lane 1) or with (lane 2) 3.5mM disuccinimidyl suberate, quenched with tris and prepared for gel electrophoresis. The resultant autoradiogram is shown.

anti-receptor antibody immunoprecipitated a protein of ca. 50 kDa from a detergent solubilized plasma membrane fraction prepared from $^{35}$S-*in vivo* labeled *N. crassa* cells (Figure 4). This protein was shown to be a phosphoprotein by immunoprecipitation of membranes from cells labeled *in vivo* with $^{32}$P-phosphate. The fact that this phosphoprotein was an active protein tyrosine kinase was demonstrated by *in vitro* kinase assay of the immunoprecipitate bound on Sepharose beads. While no autophosphorylation was observed under these conditions, this fraction was capable of phosphorylating exogenously added histone 2B. Phosphorylation was on tyrosine residues as was indicated by its alkaline stability in SDS-polyacryamide gels, and confirmed by phosphoaminoacid analysis (results not shown).

## DISCUSSION

Recent results strongly suggest that the many cellular components involved in signal transduction in mammalian systems, including G proteins, adenylate cyclase and phosphatidylinositides, exist also in unicellular eukaryotes, where they may have similar functions (reviewed in 14). We have documented the presence of specific insulin receptors and of a range of insulin induced metabolic effects in the fungus *N. crassa*

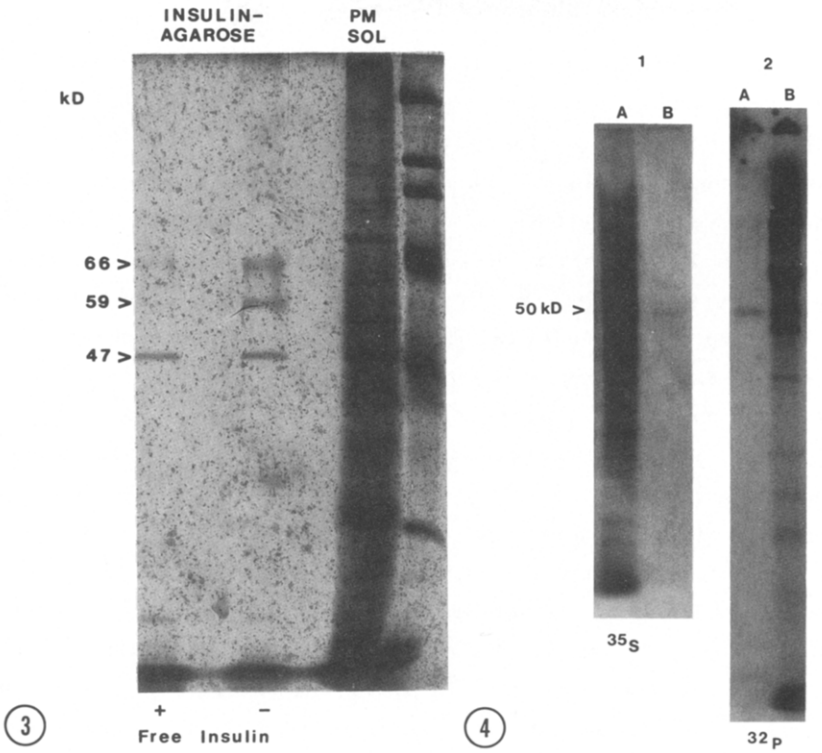


Figure 3. Affinity chromatography of solubilized *N. crassa* plasma membranes (PM sol) on an insulin-agarose (Affigel 10) matrix in presence or absence of 10 $\mu$M free insulin. After incubation, the matrices were thoroughly washed, run on SDS-PAGE, and the gel stained with silver.

Figure 4. Immunoprecipitation of an *N. crassa* protein by anti-human insulin receptor antibody. Solubilized plasma membranes were prepared from cells labeled *in vivo* with either $^{35}$S-methionine (lane 1A) or with $^{32}$P-phosphate (lane 2B) and run on SDS-PAGE. The autoradiographs are shown. Lanes 1B and 2A show the immunoprecipitates obtained from the respective preparations using anti P2.

(2,3). In this paper we have further identified the ligand specificity of the *N. crassa* insulin receptor. Further, the presence of protein tyrosine kinase activity, both in an affinity chromatography purified protein and in a protein precipitated by anti-human insulin receptor antibody is compatible with an insulin-induced, tyrosine kinase mediated signal transduction pathway in *N. crassa* cells.

The insulin receptor has been purified from a variety of different mammalian tissues and shown to consist of a tetramer of two A and two B subunits. The A subunits are extracellular glycoproteins of 125,000 to 135,000 kDa, containing the ligand binding domain. The A subunits have been preferentially cross-linked with labelled insulin in previous studies by Pilch and Czech (5). A subunits are linked to each other and to the B subunits via disulfide bonds. The B subunits are also glycoproteins, of 95,000 kDa, presumed to be membrane spanning, and containing the protein

tyrosine kinase activity of the receptor. The receptor is composed of two identical AB heterodimers which have been shown by pulse-chase experiments, and subsequently by cloning of the receptor cDNA (6), to be synthesized as a large precursor and subsequently cleaved (reviewed in 15). The fundamental role of the receptor tyrosine kinase activity in initiating intracellular events upon insulin binding seems clear (16). Recently an insulin receptor protein and its corresponding genomic sequence have been described in *Drosophila* (17,18). Like the *N. crassa* protein described here, the *Drosophila* receptor showed a specificity for insulin, and was not activated by the closely related IGF-I. The *Drosophila* receptor also possessed insulin dependent protein tyrosine kinase activity and could be precipitated by the anti-insulin receptor peptide antibody P2 (18).

The relationship between the 59-67 KDa proteins associated with specific insulin binding (Figs. 2,3) and the 50 KDa protein immunoprecipitated by anti P2 antibody (Fig. 4) remains to be determined. It is possible that they may represent two chains of an insulin receptor, by analogy with the mammalian protein (15). Alternatively, any of these proteins might be proteolytic products generated from larger proteins during purification, although proteolytic inhibitors were added in an attempt to minimize this possibility. Further insights into the *N. crassa* insulin receptor will depend upon purification of the protein(s), sequence analysis of the gene (6) and the demonstration of insulin dependent protein phosphorylation by the receptor.

## ACKNOWLEDGMENTS

Stephen E. Fawell was a fellow of the Juvenile Diabetes Association. This research was supported in part by a grant from the American Diabetes Association.

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