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ARTICLE *in* BIOCHEMISTRY · FEBRUARY 1991

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Butterworth, J. F., IV, Moran, J. R., Whitesides, G. M., & Strichartz, G. R. (1987) *J. Med. Chem. 30*, 1295-1302.
Elder, J. H., & Alexander, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4540-4544.
Escher, E. H. F., & Guillemette, G. (1978) *J. Med. Chem. 22*, 1047-1050.
Green, N. M., Konieczny, L., Toms, E. J., & Valentine, R. C. (1971) *Biochem. J. 125*, 781-791.
Hedstrom, J., Sedarous, S., & Prendergast, F. G. (1988) *Biochemistry 27*, 6304-6308.
Klueppelberg, U. G., Gaisano, H. G., Powers, S. P., & Miller, L. J. (1989a) *Biochemistry 28*, 3463-3468.
Klueppelberg, U. G., Powers, S. P., & Miller, L. J. (1989b) *Biochemistry 28*, 7124-7129.
Kurata, M., Tsunashima, Y., Iwama, M., & Kamada, K. (1975) in *Polymer Handbook*, 2nd ed. (Brandrup, J., & Immergut, E. H., Eds.) pp iv, 1-iv, 45, Wiley & Sons, New York.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Madison, L. D., Rosenzweig, S. A., & Jamieson, J. D. (1984) *J. Biol. Chem. 259*, 14818-14823.
Madison, L. D., Jamieson, J. D., & Rosenzweig, S. A. (1987) *Biochem. Biophys. Res. Commun. 143 (2)*, 761-767.
Mutter, M. (1977) *Macromolecules 10*, 1413-1414.
Naccache, P., & Sha'afi, R. I. (1973) *J. Gen. Physiol. 62*, 714-736.
Pearson, R. K., & Miller, L. J. (1987) *J. Biol. Chem. 262*, 869-876.
Pearson, R. K., Miller, L. J., Hadac, E. M., & Powers, S. P. (1987a) *J. Biol. Chem. 262*, 13850-13856.
Pearson, R. K., Powers, S. P., Hadac, E. M., Gaisano, H., & Miller, L. J. (1987b) *Biochem. Biophys. Res. Commun. 147*, 346-353.
Powers, S. P., Fourmy. D., Gaisano, H., & Miller, L. J. (1988a) *J. Biol. Chem. 263*, 5295-5300.
Powers, S. P., Pinon, D. I., & Miller, L. J. (1988b) *Int. J. Pept. Protein Res. 31*, 429-434.
Rosenzweig, S. A., Miller, L. J., & Jamieson, J. D. (1983) *J. Cell Biol. 96*, 1288-1297.
Rosenzweig, S. A., Madison, L. D., & Jamieson, J. D. (1984) *J. Cell Biol. 99*, 1110-1116.
Ruoho, A. E., & Hall, C. C. (1980) *Ann. N.Y. Acad. Sci. 346*, 90-103.
Sakamoto, C., Goldfine, I. D., & Williams, J. A. (1983) *J. Biol. Chem. 258*, 12707-12711.
Schjoldager, B., Powers, S. P., & Miller, L. J. (1988) *Am. J. Physiol. 255*, G579-G586.

Purification and Properties of a Membrane-Bound Insulin Binding Protein, a Putative Receptor, from *Neurospora crassa*†

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Received July 26, 1990; Revised Manuscript Received October 5, 1990

ABSTRACT: The protein that is responsible for specific, high-affinity binding of insulin to the surface of *Neurospora crassa* cells has been purified to homogeneity. The insulin binding activity of solubilized plasma membranes resembled that of intact cells with regard to affinity of binding, specificity for mammalian insulins, and amount of insulin bound per cell. Insulin binding activity was purified from Triton X-100 solubilized membranes in two steps: FPLC on a MonoQ HR5/5 column; and affinity chromatography on insulin-agarose. The pure material migrated as a single band of ca. 66 kDa on SDS gels, pI = 7.4 by isoelectric focusing. The protein bound 5.34 pmol of insulin/μg, or 35% of that expected for univalent binding. Cross-linking of ^{125}I-insulin to pure protein or to solubilized membranes revealed a single labeled band of 67-70 kDa on SDS gels. In nonreducing native gels, two labeled bands of ca. 55 and 110 kDa were produced after cross-linking, and two bands of similar molecular weight bound iodinated insulin after transfer to nitrocellulose filters. These may correspond to active monomer and dimer forms. The pure protein possessed no protein kinase activity against itself, or against exogenous substrates (histone H_2, casein, or the synthetic peptide Glu^{80}-Tyr^{20}), and possessed no detectable phosphorylated amino acids. It is suggested, however, that this 66-kDa protein is the "receptor" that mediates insulin-induced downstream metabolic effects.

Previous reports from this laboratory have documented several metabolic effects of mammalian insulin upon *Neurospora crassa* cells: enhanced production of glucose metabolites including CO_2, ethanol, alanine, and glycogen; enhanced growth and improved viability; and enhanced accumulation of intracellular sodium upon addition of glucose (Fawell et al., 1988; Greenfield et al., 1988, 1990; McKenzie et al., 1988). These observations were of special interest in the light of numerous findings by others that a biologically active insulin-like molecule was found in several different microbial eukaryotes (LeRoith et al., 1980, 1985), in insects, invertebrates, and plants (Collier et al., 1987; Conlon et al.,

†Supported by Grant DK39502 from the National Institutes of Health.
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0006-2960/91/0430-682$02.50/0

1985; DePablo et al., 1988; Kawakami et al., 1989; Maier et al., 1988; Robitzki et al., 1989), and in prokaryotes as well (LeRoith et al., 1981; Rubinovitz & Shiloach, 1985). They were also consistent with reports of an insulin-dependent decrease in adenylate cyclase activity in *N. crassa* membranes (Flawia & Torres, 1973).

When the insulin-induced enhancement of glycogen synthesis in *N. crassa* was examined in greater detail, it was discovered that the mechanism resembled that in mammalian cells, in that the enzyme glycogen synthase was converted from a glucose 6-phosphate dependent to an independent form by insulin (Fawell et al., 1988). In mammalian cells, this is accomplished by an insulin-dependent dephosphorylation of the enzyme (Cohen et al., 1985; Miller, 1985; Soderlin & Sheorain, 1985), reflecting the culmination of a signal transduction pathway that begins when insulin binds to the membrane-bound insulin receptor (Morgan et al., 1986; Rosen, 1987). Two additional findings were consistent with the idea that a similar mechanism was operating in *N. crassa* cells: (i) the *N. crassa* glycogen synthase is a phosphoenzyme whose phosphorylation state governs its activity (Tellez-Inon et al., 1969); and (ii) *N. crassa* cells possess specific, saturable, high-affinity binding sites for insulin on their surface (Fawell & Lenard, 1988; McKenzie et al., 1988). As a first step in the study of this putative signal transduction pathway, we have purified and characterized the membrane protein that is responsible for insulin binding by *N. crassa* cells.

Materials and Methods

Materials. Human recombinant IGF-I[1] and IGF-II were obtained from Boehringer Mannheim. Monoclonal anti-phosphotyrosine antibodies (PY20IgG2a and PY69IgG2b) were obtained from ICN. Porcine proinsulin was a gift from Ron Chance (Eli Lilly Research Laboratories, IN). Affi-Gel 10 was purchased from Bio-Rad. Radiolabeled materials were purchased from NEN. All other reagents were purchased from Sigma. Triton X-100, reduced (hydrogenated to minimize UV absorption), was used exclusively.

Cells. Strain FGSC 4761 (Fungal Genetic Stock Center, Arcata, CA) is a rapidly growing variant of the wall-less slime mutant FGSC 1118 of *N. crassa* (Scarborough, 1985). Cells were maintained by daily passage in an undefined medium, SeM (Schulte & Scarborough, 1975). For most of the experiments described in this paper, cells were grown in a rich defined medium, SDM (McKenzie et al., 1988). Cells grown in SDM possessed substantially greater insulin binding activity than cells grown in SeM or in minimal medium (S. Fawell, unpublished observations).

Buffers. Buffers used were as follows: (A) 50 mM HEPES, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM DTT, 0.2 mM PMSF, 10 μg/mL aprotinin, 20 μg/mL soybean trypsin inhibitor, and 5 μg/mL leupeptin; (B) 50 mM HEPES, pH 7.4, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL aprotinin, 20 μg/mL soybean trypsin inhibitor, and 5 μg/mL leupeptin; (C) 50 mM HEPES, pH 7.4, 10% glycerol, 150 mM NaCl, 10 mM $MgCl_2$, and 0.2 mM PMSF; (CTX) buffer C containing 0.1% TX; (D) 50 mM HEPES, pH 7.4, 0.5 M NaCl, 0.2 mM PMSF, and 0.1% TX; (E) 100 mM HEPES, pH 7.9, 0.12 M NaCl, 1.2 mM $MgCl_2$, 2.5 mM KCl, 1 mM EDTA, 0.5 mM sodium acetate, 1 mg/mL bacitracin, 1 mg/mL BSA, and 0.1% TX; (F) 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mg/mL bacitracin, 0.5 mg/mL BSA, 100 μM sodium orthovanadate; (PBS) 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO4, 1.5 mM KH_2PO4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$; and (TBST) 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20.

Preparation of N. crassa Membranes. N. crassa cells grown overnight at 30 °C in SeM (300 mL) were used to inoculate 12 L of SDM in a fermentor. Growth was allowed to continue for 22–24 h at 30 °C, with the fermentor maintained at a set point above 20% dissolved O_2, maintained via control of air flow and agitation. Cells were harvested by centrifugation (1500 rpm for 10 min) at 4 °C, and the cell pellet was washed twice with 20 mM HEPES, pH 7.4, and 150 mM NaCl. Washed cells (270–290 g wet weight) were suspended in 200 mL of buffer A and homogenized by passing through a French press cell disintegrator at 14000 psi. Cell debris was removed by centrifugation (8000 rpm, 20 min, 4 °C) in a Sorvall GSA rotor. The supernatant was centrifuged at 100000*g* for 60 min at 4 °C. The pellet was suspended in 10 volumes of buffer B, homogenized by five passes of a tight fitting pestle in a Dounce homogenizer, and centrifuged again at 100000*g* for 60 min at 4 °C. The sedimented plasma membranes were resuspended in 5 volumes of buffer B and stored at −80 °C. Further purification was obtained by resuspending the membranes in buffer B at 10 mg of protein/mL and centrifuging onto a cushion of 35% sucrose in buffer B at 18000 rpm for 60 min at 4 °C in an SW 27 rotor. The dense white interphase material was recentrifuged at 100000*g* for 60 min at 4 °C. The pellet was resuspended in buffer C (25–30 mg of protein/mL) and stored at −80 °C.

Purification of Insulin Binding Protein. All steps were done at 0–4 °C unless stated otherwise. Purified membranes were solubilized in buffer C containing 2% TX for 60 min with end-over-end agitation, and the protein concentration was adjusted to 10–12 mg/mL. A clear superantant was obtained by centrifugation at 100000*g* for 90 min.

The MonoQ HR5/5 anion-exchange column was pretreated with buffer CTX containing 1 M NaCl (high-salt buffer) and then equilibrated with buffer CTX (low-salt buffer). Solubilized membranes were loaded onto the column (flow rate 1 mL/min), and the column was washed with low-salt buffer. The column was eluted with a linear gradient (30 mL total) of 0.15–1 M NaCl in buffer CTX. Fractions (1 mL) were collected and assayed for binding activity. Due to the limited capacity of the MonoQ column, this procedure was repeated 8–10 times in order to pass through all the solubilized membranes prepared from a batch of cells prepared as described above.

An insulin affinity column was prepared by coupling porcine insulin to Affi-Gel 10 as previously described (Fawell & Lenard, 1988), except that the urea was omitted from the coupling buffer. The active fractions from the MonoQ column were pooled and applied over the course of 1 h at room temperature to a 4-mL insulin–agarose column that had been preequilibrated in buffer D. The column was washed in buffer D and eluted with sodium acetate buffer pH 5.5, as described (Petruzelli et al., 1986). Fractions (1 mL) were collected into tubes containing 60 μL of 1 M HEPES, pH 9.0, in 0.1% TX. Activity was tested immediately. The first five fractions contained most of the activity. Active fractions were aliquoted and stored at −80 °C.

Insulin Binding Assay. Insulin binding activity was measured by incubating the receptor preparation at 15 °C for 3–4 h with ^{125}I-porcine insulin (100 pM, 200 cpm/fmol) in a final volume of 0.4 mL of buffer E in the absence or presence of excess unlabeled porcine insulin (10 μM). In the competition

[1] Abbreviations: TX, Triton X-100, reduced; PMSF, phenylmethanesulfonyl fluoride; IGF, insulin-like growth factor; DTT, DL-dithiothreitol.

binding experiments, various amounts (100 pM–10 μM) of unlabeled insulins, proinsulin, or IGFs were added. The receptor–insulin complex was separated from free insulin by poly(ethylene glycol) precipitation (Cuatrecasas, 1972). Assays were performed in duplicate, and data were corrected for nonspecific binding.

Protein Kinase Assay. Autophosphorylation of insulin binding protein and phosphorylation of the exogenous substrates casein, histone H_2, and the synthetic polypeptide Glu^{80}–Tyr^{20} were addressed with various conditions in the presence and absence of porcine insulin (Kole et al., 1988): (1) Protein was preincubated in buffer F for 10 min at 22 °C and 10 min in ice in the presence of 0, 10, 50, 100, 250, or 500 nM porcine insulin. The phosphorylating reaction was initiated by the addition of either 10 or 500 μM [γ-^{32}P]ATP. The reactions were terminated after a 30-min incubation at 0 °C by adding SDS sample buffer. The samples were electrophoresed on a 10% SDS–polyacrylamide gel. The gel was dried and autoradiography was performed with Kodak XAR-5. (2) Protein in the presence and absence of 100 nM insulin was preincubated in buffer F for 10 min at 22 °C, and different concentrations of [γ-^{32}P]ATP (e.g., 10 μM, 100 μM, 500 μM, or 1 mM) were added to initiate the reaction. The reaction was terminated after 30 min at 22 °C by adding SDS sample buffer and processing as above. (3) Protein was preincubated with or without 100 nM insulin for 10 min in buffer F containing different concentrations of $MnCl_2$ (e.g., 1, 2, 4 or 10 mM). Reaction was started by adding 10 μM [γ-^{32}P]ATP and terminated after 10 or 30 min at 22 °C by adding SDS sample buffer.

Protein Determinations. Protein concentration was measured according to the method of Bradford (1976) or modified Lowry procedure (Bensadoun & Weinstein, 1976), using BSA as standard. Following affinity chromatography, different concentations of receptor protein were compared with known concentrations of BSA in silver-stained SDS–polyacrylamide gels.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed in 10% gels containing 0.1% SDS according to the procedure of Laemmli (1970). For nonreducing native gels, SDS and β-mercaptoethanol were omitted from gels and buffers. Two-dimensional isoelectric focusing–SDS gel electrophoresis was performed as described by Sanders et al. (1980). Proteins were visualized by Coomassie Blue or silver staining (Switzer et al., 1979).

Cross-Linking Experiments. Intact membranes or solubilized proteins were incubated in the absence or presence of 10 μM porcine insulin for 30 min at 23 °C in buffer E lacking BSA, and then ^{125}I-insulin (10^3 cpm/fmol) was added to a final concentration of 4 nM. After incubation for 4 h at 15 °C, disuccinimidyl suberate to a final concentration of 0.3 mM was added, and the reaction allowed to proceed for 30 min at 4 °C. The reaction was quenched by the addition of 5 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, and the samples were analyzed by autoradiography of reducing and nonreducing gels.

^{125}I-Insulin Blotting. Proteins separated on reducing or nonreducing native gels were electrophoretically transferred to nitrocellulose papers by using Tris–glycine buffer without methanol (Pluskal et al., 1986), and the nitrocellulose papers were treated with 5% nonfat dry milk in PBS for 16 h at 4 °C. The papers were then treated with 20 mL of 5% nonfat dry milk in PBS containing ^{125}I-insulin (10^6 total cpm) in the presence or absence of excess unlabeled insulin (10 μM) for 2 h at room temperature. The unbound ^{125}I-insulin was washed off with 5% nonfat dry milk in PBS, and the papers were dried and autoradiographed.

^{125}I-Insulin Degradation Assay. Insulin-degrading activity was determined as described by Shii et al. (1985) with modifications. Briefly, enzyme was incubated in 200 μL of buffer E in the presence or absence of bacitracin. The reaction was started by the addition of 1 nM ^{125}I-insulin. After 30 min at 37 °C, the reaction was stopped with 200 μL of 10% TCA, incubated 15 min on ice, and centrifuged in a Beckman microfuge for 10 min. Radioactivity in both pellet and supernatant were determined in an LKB compuGamma CS γ counter. The relative amount of released radioactivity in the soluble fraction was determined as described (Garcia et al., 1988).

^{32}P-Labeling of Cells and Immunoblotting of Membrane Phosphoproteins with Anti-Phosphotyrosine Antibodies. An overnight culture (50 mL) of *N. crassa* cells in low-phosphate (75 μM) SDM medium was labeled for 3 h at 30 °C with [^{32}P]P_i (200 μCi/mL), and then insulin (10 nM final concentration) or an equal volume of buffer was added. After 10 min of incubation, cells were washed in low-phosphate SDM medium, suspended in buffer A containing 100 μM sodium orthovanadate, and homogenized 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 100000*g*. The pellet, a crude plasma membrane fraction, was suspended into buffer B containing 100 μM sodium orthovanadate. The proteins from two-dimensional gels were transferred to Immobilon-P membranes by electrophoresis for 12 h at 40 V (Pluskal et al., 1986). The PVDF blots were then stained with Ponceau S for 3 min to check the efficiency of transfer and subsequently destained in distilled water. The blot was incubated overnight at 4 °C in a blocking solution containing 3% BSA in TBST. The blots were then incubated with 1:1000 dilution of anti-phosphotyrosine antibody, either PY69 or PY20, or 1 h and washed several times with TBST containing 1% BSA. Binding was visualized by use of anti-mouse IgG conjugated with alkaline phosphatase (Blake et al., 1984). Air-dried blots were then autoradiographed.

Results

In order to purify the insulin binding protein from *N. crassa* membranes, it was first necessary to optimize the measurement of insulin binding in TX-solubilized membrane preparations. The optimal pH for the binding assay was found to be in the range of 7.0–7.9; specific binding decreased above this range, while nonspecific binding increased below it. Both HEPES and MOPS proved to be suitable buffers for binding, but binding was decreased if Tris or phosphate were used. Optimized assay conditions are given under Materials and Methods.

The amount of insulin bound increased linearly over at least a 10-fold range of TX solubilized membrane protein (Figure 1). Insulin binding activity was estimated from these data to be on the order of 10^3 binding sites/cell. Unlabeled bovine and porcine insulins compete equally effectively with ^{125}I-insulin for binding, with 50% displacement occurring at 50–80 nM. In contrast, IGF-I displaces ^{125}I-insulin only weakly, while IGF-II and proinsulin are practically ineffective (Figure 2). Both insulin binding affinity and specificity of solubilized membranes correspond closely to those previously found for intact *N. crassa* cells (Fawell & Lenard, 1988).

Table I summarizes the results of a typical receptor purification, starting with crude membranes from a 12-L culture of *N. crassa* cells (see Materials and Methods for details).

Table I: Purification of *N. crassa* Insulin Receptor

step	total protein (mg)	specific insulin binding activity (pmol/mg)	total activity (pmol)	purification, *x*-fold	yield (%)
(1) crude *N. crassa* membranes	1952	0.037	72.2	1	100
(2) sucrose-purified membranes	647	0.090	58.2	2.4	81
(3) supernatant, TX-solubilized membranes	325	0.16	52.0	4.3	72
(4) MonoQ ion-exchange column	2.4	16.10	38.6	435.0	54
(5) insulin–agarose	0.005	5,340	26.7	144 324	37

[a] Insulin binding activity was measured as described under Materials and Methods. All assays were performed in duplicate, and the data have been corrected for nonspecific binding.

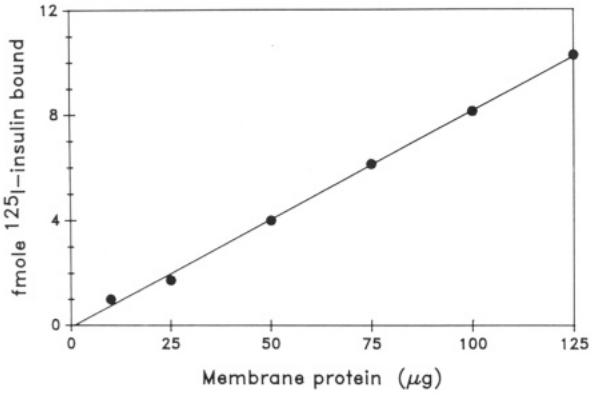

FIGURE 1: Specific binding of ^{125}I-insulin to TX-solubilized *N. crassa* membranes. Assay was as described under Materials and Methods, except that ^{125}I-insulin concentration was 400 pM, and incubation was at 4 °C for 16 h.

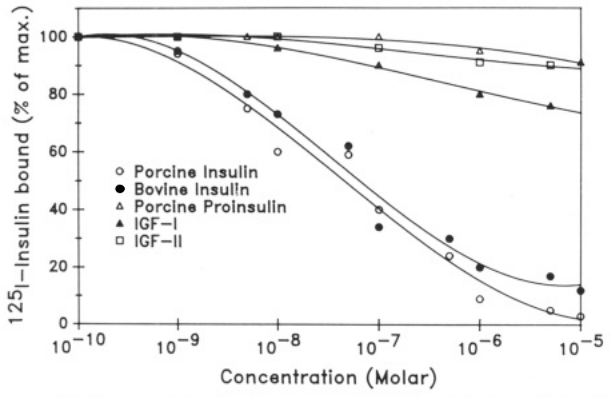

FIGURE 2: Competitive displacement of ^{125}I-insulin from TX-solubilized *N. crassa* membranes (100-μg aliquots) by insulin and insulin analogues.

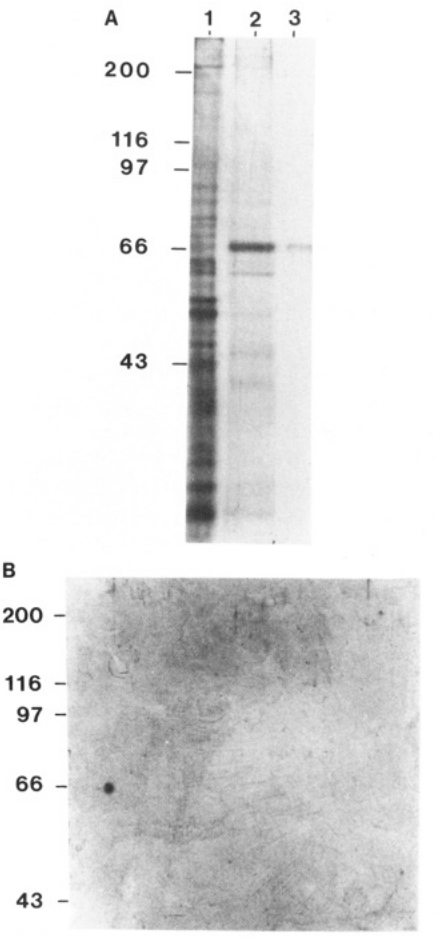

FIGURE 3: (A) SDS–polyacrylamide gel electrophoresis of (lane 1) TX-solubilized, sucrose-purified membranes; (lane 2) pooled insulin binding fractions from the MonoQ column; (lane 3) eluate from the insulin–agarose affinity column. (B) Two-dimensional gel electrophoresis of purified insulin binding protein. Gels were stained with silver.

Crude membranes (step 1) contain 75–80% of the total insulin binding activity of disrupted cells. No insulin binding activity was observed in cytoplasmic fractions. Modest enrichments of specific activity were obtained by centrifuging of the crude membranes onto a sucrose cushion (step 2), and from clarification of the TX-solubilized preparation (step 3). The major purification on the MonoQ column (step 4) resulted from removal of other proteins in the low-salt (0.15 M. NaCl) flow-through. Most of the insulin binding activity eluted in a peak between 0.38 and 0.42 M NaCl. The pooled active material was then allowed to bind to an insulin–agarose column and eluted at low pH (step 5) to yield ca. 5 μg of protein. SDS gel electrophoresis showed a single band of ca. 66 kDa (Figure 3A). Two-dimensional gel electrophoresis revealed a single spot of ca. 66 kDa, with a p*I* of ca. 7.4 (Figure 3B). Purified protein has specific insulin binding activity of 5.34 pmol/μg, or 35% of the expected value if 1 mol of insulin is bound per mole of 66-kDa binding protein.

The binding protein is quite stable in the crude plasma membrane extract. However, following purification on insulin–agarose, activity was rapidly destroyed by freezing and thawing. This could be partially prevented by quick-freezing in 20% glycerol.

Confirmation that this purified protein actually corresponded to the insulin binding protein was obtained from cross-linking and ligand binding experiments. Results of cross-linking experiments are shown in Figure 4. A single band of 67–70 kDa was seen on SDS gels after cross-linking of ^{125}I-insulin to solubilized membranes (Figure 4A, lanes 1 and 2), to the MonoQ-purified material (lanes 3 and 4), or to the pure protein (lanes 5 and 6). Cross-linked material was observed in the absence of excess unlabeled insulin (lanes 1, 3 and 5) but not in its presence (lanes 2, 4, and 6). When the same preparations were subjected to electrophoresis under nonreducing conditions in the absence of SDS, specifically labeled bands were seen at ca. 55 and 110 kDa (Figure 4B). It seems most likely that these correspond to active monomeric and dimeric forms of the binding protein. The specificity of binding was retained by the affinity-purified material, as shown

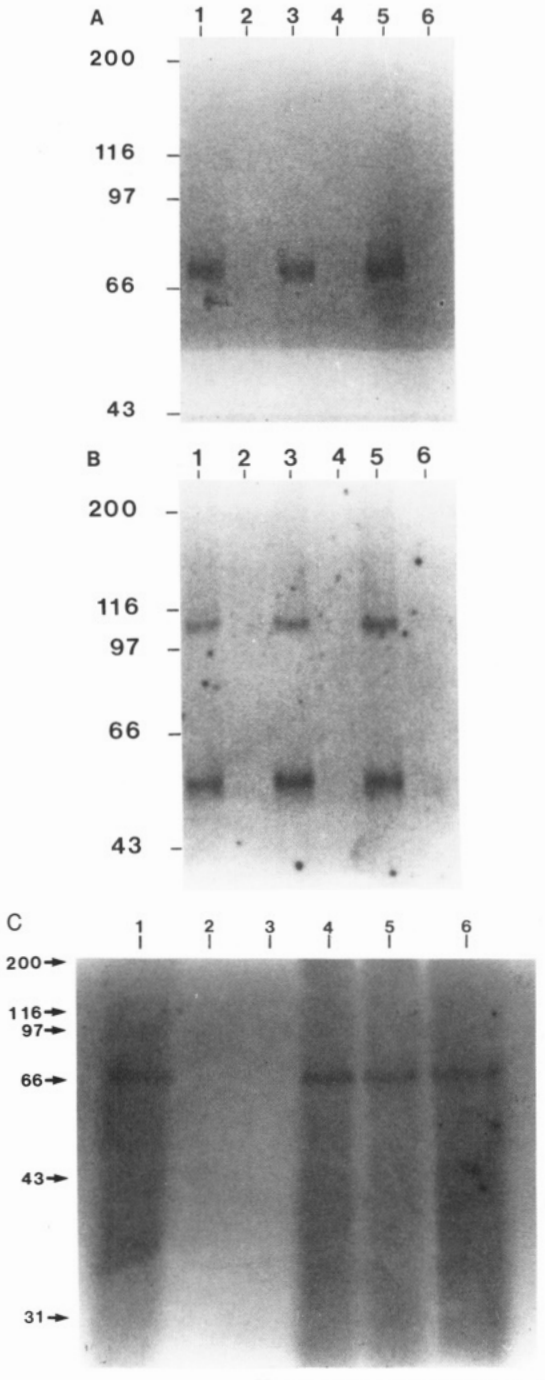

FIGURE 4: Cross-linking of ^{125}I-insulin to solubilized *N. crassa* membrane proteins in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 10 μM unlabeled porcine insulin. (A) Reducing gel. (B) Nonreducing native gel. (Samples of molecular weight standards contained SDS.) Lanes 1 and 2: TX-solubilized, sucrose-purified membranes (50 μg). Lanes 3 and 4: Pooled insulin binding fractions from the MonoQ column (2 μg). Lanes 5 and 6: Eluate from the insulin–agarose affinity column (100 ng). Exposure of the film was for 21 days. (C) Displacement of ^{125}I-insulin from affinity-purified binding protein by insulin and related peptides (reducing gel). (Lane 1) No addition; (lane 2) 10 μM porcine insulin; (lane 3) 10 μM bovine insulin; (lane 4) 10 μM IGF-I; (lane 5) 10 μM IGF-II; (lane 6) 10 μM proinsulin.

in Figure 4C. ^{125}I-Insulin was displaced from the protein by porcine or bovine insulin (Figure 4C, lanes 2 and 3) but not by IGF-I (lane 4), IGF-II (lane 5), or proinsulin (lane 6).

Proteins of similar molecular weight bound specifically to ^{125}I-insulin after transfer of membrane protein preparations from nonreducing native gels to nitrocellulose paper (Figure 5); once again, binding was observed only in the absence of

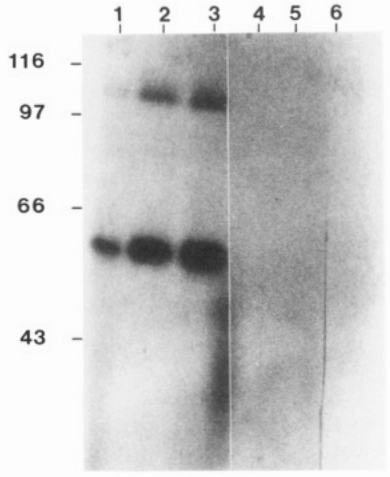

FIGURE 5: Binding of ^{125}I-insulin to *N. crassa* membrane proteins in the absence (lanes 1–3) or presence (lanes 4–6) of 10 μM unlabeled insulin. Samples were separated on nonreducing native gels and transferred to nitrocellulose paper as described under Materials and Methods. Samples of molecular weight standards contained SDS. Lanes 1 and 4: TX-solubilized, sucrose-purified membranes (50 μg). Lanes 2 and 5: Pooled insulin binding fractions from the MonoQ column (2 μg). Lanes 3 and 6: Eluate from the insulin–agarose affinity column (0.3 μg). Exposure of the film was for 30 days.

excess unlabeled insulin (cf. lanes 1–3 and 4–6). Blotting of a two-dimensional gel run under reducing but nondenaturing conditions revealed a single spot of ^{125}I-insulin binding, at a position very close to the silver-stained spot down in Figure 3B; binding of ^{125}I-insulin did not occur in the presence of excess unlabeled insulin (data not shown). No binding of ^{125}I-insulin could be detected after blotting of membrane proteins from denaturing gels.

The possibility was considered that this protein might represent an insulin-degrading enzyme such as those that have been isolated from mammalian (Duckworth et al., 1972; Shii et al., 1986) and *Drosophila* (Garcia et al., 1988) cells. We found, in fact, that *N. crassa* membranes do possess a bacitracin-inhibitable insulin degrading activity. However, that activity was separated from the 66-kDa insulin binding protein during chromatographic purification. When the solubilized membrane preparation was chromatographed on a MonoQ column (as described under Materials and Methods), the insulin degrading activity was found exclusively in the flow-through fractions, while the 66-kDa binding protein was retained on the column and subsequently eluted in 0.38–0.42 M NaCl, as described above. This fraction was devoid of measurable insulin degrading activity.

Protein kinase activity was absent from the purified protein preparation. Neither autophosphorylation of the receptors nor phosphorylation of exogenous substrates, viz., histone H_2, casein, or Glu^{80}–Tyr^{20}, was found, either in the presence or in the absence of added insulin, under a wide variety of experimental conditions (see Materials and Methods).

In order to analyze the in vivo autophosphorylation status of the binding protein, cells were labeled with [^{32}P]P_i in the presence or absence of insulin, and phosphorylated proteins were analyzed by two-dimensional gel electrophoresis, western blotting with anti-phosphotyrosine antibodies, and subsequent autoradiography. Immunoblot with PY20 or PY69 did not show any phosphotyrosine-containing protein in the region of the binding protein; i.e., molecular mass 66 kDa and p*I* 7.4 (data not shown). Figure 6 is an example autoradiogram obtained from the PY69 immunoblot. It indicates that there is no phosphorylation of proteins in the region of molecular mass 66 kDa and p*I* 7.4.

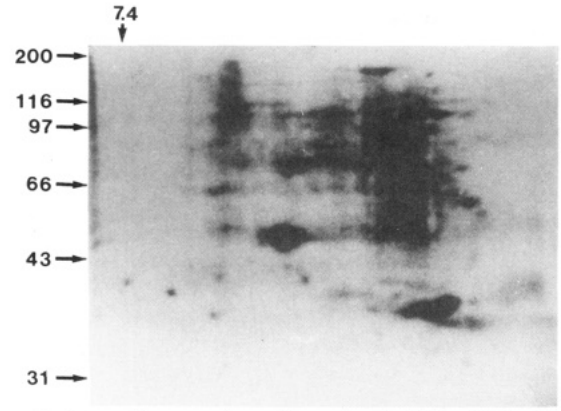

FIGURE 6: Autoradiogram of two-dimensional gel blot obtained from a crude *N. crassa* membrane fraction after labeling in vivo with [^{32}P]P_i in the presence of insulin (10 nM), as described under Materials and Methods.

Discussion

The protein described in this report appears to account for the great majority of the insulin binding activity of whole *N. crassa* cells. From the binding capacity of solubilized membranes (Figure 1), a density of ca. 10^3 sites/cell was estimated, while previously published Scatchard plots using intact cells indicated the presence of 100–3500 insulin binding sites/cell (McKenzie et al., 1988). Further, both cross-linking and blotting experiments which compared purified insulin binding protein with unfractionated membranes (Figures 4 and 5) or whole cells (Fawell & Lenard, 1988) detected only a single insulin binding protein in each case.

It was reported in a previous paper that two protein bands from solubilized *N. crassa* membranes, of 66 and 59 kDa, were specifically bound to an insulin–agarose column (Fawell & Lenard, 1988). In the present paper, only material eluted from such columns was analyzed, and no evidence of a 59-kDa protein was found. The 59-kDa band might thus represent an additional insulin binding protein that is not eluted from the column under the conditions used here, or it might be a proteolytic fragment of the 66 kDa protein characterized in this paper. It should be noted that no cross-linking of insulin to a 59-kDa band was seen, either in solubilized membrane preparations (Figure 4A) or with intact cells (Fawell & Lenard, 1988).

There are strong, albeit still circumstantial, arguments that this 66-kDa protein represents an insulin receptor in the classical sense, i.e., a protein that initiates a series of functionally important intracellular signals upon binding a ligand. First, *N. crassa* cells secrete a molecule that resembles insulin both immunologically and functionally. The insulin-like molecule was detected by radioimmunoassay using antibodies prepared against mammalian insulin (LeRoith et al., 1980), although not all anti-insulin antibodies are capable of detecting it (M. McKenzie, personal communication). The partially purified material was shown to possess insulin-like activity when tested against rodent adipocytes (LeRoith et al., 1980). A similar material has been found in other microbial eukaryotes and, remarkably, in prokaryotes as well (Le Roith et al., 1980, 1981, 1985; Rubinovitz & Shiloach, 1985). Second, treatment of *N. crassa* cells with bovine, porcine, or human insulin has been shown to elicit a variety of metabolic effects that are strongly reminiscent of insulin effects of mammalian cells. These include enhanced consumption of glucose, enhanced production of CO_2, ethanol, alanine, and glycogen and other metabolites from glucose, increased retention of intracellular sodium during the course of glucose consumption (Fawell et al., 1988; Greenfield et al., 1988, 1990; McKenzie et al., 1988), and decreased adenylate cyclase activity by isolated membranes (Flawia & Torres, 1973).

Of particular interest for the present discussion is the effect of insulin in glycogen synthesis (Fawell et al., 1988). In insulin-treated *N. crassa* cells the enzyme glycogen synthase was shown to undergo a conversion from a glucose 6-phosphate dependent (D form) to a glucose 6-phosphate independent (I) form within 30 min of adding 100 nM insulin. Half-maximal effect was achieved with 2 nM insulin. This effect is identical with that seen in mammalian cells, in which the D to I conversion of glycogen synthase is accomplished by dephosphorylation of the enzyme, induced by an insulin-specific phosphatase (Cohen et al., 1985; Miller, 1985; Soderlin & Sheorain, 1985). Although a similar mechanism has not yet been demonstrated in *N. crassa* cells, the *N. crassa* glycogen synthase is known to be a phosphoenzyme that can be activated by dephosphorylation (Tellez-Inon et al., 1969). These remarkable similarities between mammalian and fungal effects of insulin strongly suggest the existence in fungi of a signal transduction pathway, initiated by insulin binding to a specific plasma membrane protein.

The *N. crassa* insulin binding protein described here bears little resemblance to the well-characterized insulin receptors of mammals and *Drosophila*. These receptors consist of two copies of each of polypeptides of 95 and 110–135 kDa (Rosen, 1987). The 95-kDa chain spans the membrane and possesses tyrosine kinase activity in its cytoplasmic domain, while the larger chain is entirely external and governs insulin binding activity. The *N. crassa* protein, on the other hand, is only 66 kDa and possesses no detectable kinase activity. It is possible, therefore, that a kinase activity may have been inactivated or proteolytically removed during purification of the insulin binding protein. Alternatively, ligand binding may activate a kinase activity by noncovalent association; indeed, we have found that insulin enhanced the phosphorylation of specific proteins, in *N. crassa* cells and membranes, at both serine/threonine and tyrosine residues (Kole & Lenard, 1990). The role of the 66-kDa insulin binding protein in this response remains to be determined.

Acknowledgments

The expert technical assistance of Roger Vanderoef is gratefully acknowledged.

Registry No. Insulin, 9004-10-8; insulin receptor protein kinase, 88201-45-0.

References

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Blake, M. S., Johnston, K. W., Russel-Jones, G. J., & Gotschlicht, E. C. (1984) *Anal. Biochem. 136*, 175–179.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cohen, P., Parker, P. J., & Woodgett, J. R. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 213–233, Plenum Press, New York.

Collier, E., Watkinson, A., Cleland, C. F., & Roth, J. (1987) *J. Biol. Chem. 262*, 6238–6247.

Conlon, J. M., Reinecke, M., Thorndyke, M. C., & Falkmer, S. (1988) *Horm. Metab. Res. 20*, 406–410.

Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 318–322.

DePablo, F., Chambers, S. A., & Ota, A. (1988) *Dev. Biol. 130*, 304–310.

Duckworth, W. C., Heinemann, M. A., & Kitabchi, A. E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3698–3702.

Fawell, S. E., & Lenard, J. (1988) *Biochem. Biophys. Res. Commun. 155*, 59-65.
Fawell, S. E., McKenzie, M. A., Greenfield, N. J., Adebodun, F., Jordan, F., & Lenard, J. (1988) *Endocrinology 122*, 518-523.
Flawia, M. M., & Torres, H. N. (1973) *J. Biol. Chem. 248*, 4517-4520.
Garcia, J. V., Fenton, B. W., & Rosner, M. R. (1988) *Biochemistry 27*, 4237-4244.
Greenfield, N. J., McKenzie, M. A., Adebodun, F., Jordan, F., & Lenard, J. (1988) *Biochemistry 27*, 8526-8533.
Greenfield, N. J., Cherapak, C. N., Adebodun, F., Jordan, F., & Lenard, J. (1990) *Biochim. Biophys. Acta 1025*, 15-20.
Kawakami, A., Iwami, M., Nagasawa, H., Suzuki, A., & Ishizaki, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6843-6847.
Kole, H. K., & Lenard, J. (1990) *J. Cell Biol. 111*, 472a.
Kole, H. K., Abdel-Ghany, M., & Racker, E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5849-5853.
Laemmli, U. K. (1970) *Nature 227*, 680-686.
LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6184-6188.
LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1981) *J. Biol. Chem. 256*, 6533-6536.
LeRoith, D., Shiloach, J., Heffron, R., Rubinovitz, C., Tanenbaum, R., & Roth, J. (1985) *Can. J. Biochem. Cell Biol. 63*, 839-849.
Maier, V., Steiner, P., Fuchs, J., Pfeifle, B., Mezger, M., & Pfeiffer, F. (1988) *Horm. Metab. Res. 20*, 421-425.
McKenzie, M. A., Fawell, S. E., Cha, M., & Lenard, J. (1988) *Endocrinology 122*, 511-517.
Miller, T. J. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 247-261, Plenum Press, New York.
Morgan, D. O., Ho, L., Korn, L. J., & Roth, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 328-332.
Petruzzelli, L., Herrera, R., & Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327-3331.
Pluskal, M. G., Przekop, M. B., Kavonian, M. R., Vecoli, C., & Hicks, D. A. (1986) *Biotechniques 4*, 272-283.
Robitzki, A., Schröder, H. C., Vgarkovic, D., Pfeifer, K., Uhlenbruck, G., & Müller, W. E. G. (1989) *EMBO J. 8*, 2905-2909.
Rosen, O. M. (1987) *Science 237*, 1451-1458.
Rubinovitz, C., & Shiloach, J. (1985) *FEMS Microbiol. Lett. 29*, 53-58.
Sanders, M. M., Groppi, V. E., & Browning, E. T. (1980) *Anal. Biochem. 103*, 157-165.
Scarborough, G. A. (1985) *Exp. Mycol. 9*, 275-278.
Schulte, T. H., & Scarborough, G. A. (1975) *J. Bacteriol. 122*, 1076-1080.
Shii, K., Baba, S., Yokono, L., & Roth, R. A. (1985) *J. Biol. Chem. 260*, 6503-6506.
Shii, K., Yokono, K., Baba, S., & Roth, R. A. (1986) *Diabetes 35*, 675-682.
Soderlin, T. R., & Sheorain, V. S. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 235-245, Plenum Press, New York.
Switzer, R. C., Merril, C. R., & Shifrin, S. (1979) *Anal. Biochem. 98*, 231-237.
Tellez-Inon, M. T., Terenzi, H., & Torres, H. N. (1969) *Biochim. Biophys. Acta 191*, 765-768.

Resonance Raman Spectroscopy of Bilirubins: Band Assignments and Application to Bilirubin/Lipid Complexation†

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ABSTRACT: Resonance Raman spectra of bilirubins IXα, IIIα, and XIIIα and mesobilirubin XIIIα in alkaline aqueous and chloroform solutions are reported. Partial band assignments of bilirubin IXα are proposed. The model compounds confirm assignments of bands of the Raman spectrum of bilirubin IXα to each of the two different pyrromethenones. Resonance Raman spectra of mesobilirubin IVα, vinylneoxanthobilirubinic acid, and vinylisoneoxanthobilirubinic acid in alkaline aqueous solution and of the tetra-*n*-butylammonium salt of bilirubin IXα are used to define markers for the presence or absence of internal hydrogen bonds. Interaction of bilirubin dianion and sphingomyelin liposomes is studied. The Raman evidence suggests that in the bilirubin dianion/liposome complex the intramolecular hydrogen bonds between the propionate groups and the lactam NH/CO are ruptured. It is proposed that in the complex the bilirubin propionates form ion pairs with the quaternary ammonium ion of the choline moiety of sphingomyelin.

(4*Z*,15*Z*)-Bilirubin IXα (BR IXα), commonly called bilirubin (BR), is formed in vivo from enzyme-catalyzed ring opening of heme proteins. The molecule generally adopts an internally hydrogen-bonded conformation as shown in structure **1a**. The neutral molecule is sparingly water soluble, although the dipropionate anion (structure **1b**) is soluble in alkaline solution.

BR has two similar planar pyrromethenone groups, A-B and C-D. Lactam ring A and pyrrole B are in the *Z* con-

†This work was supported in part by NIH Grants GM-26001 (to M.D.M.) and HD17779 (to D.A.L.).

0006-2960/91/0430-688$02.50/0