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The β Subunit of the Insulin Receptor Is an Insulin-Activated Protein Kinase†

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ABSTRACT: In the presence of adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP) and a partially purified human placental insulin receptor preparation, insulin stimulates the phosphorylation of an M_r 94 000 protein in a time- and dose-dependent manner. Half-maximal stimulation of ^{32}P incorporation occurs at (2–3) × 10^{-9} M insulin, a concentration identical with the K_d for insulin binding in this preparation. Immunoprecipitations with monoclonal anti-insulin receptor antibody demonstrate that the M_r 94 000 protein kinase substrate is a component of the insulin receptor, the β subunit. If the partially purified, soluble placental receptor preparation is immunoprecipitated and then exposed to [γ-^{32}P]ATP and insulin, phosphorylation of the M_r 94 000 protein is maintained. The photoincorporation of 8-azido[α-^{32}P]ATP into placental insulin receptor preparations was carried out to identify the ATP binding site responsible for the protein kinase activity. Photoincorporation into numerous proteins was observed, including both subunits of the insulin receptor. However, when photolabeling was performed in the presence of excess adenosine 5′-(β,γ-imidotriphosphate), a nonhydrolyzable ATP derivative, the β subunit of the insulin receptor was the only species protected from label incorporation. These data indicate that the β subunit of the insulin receptor has insulin-dependent protein kinase activity. Phosphotyrosine formation is the primary result of this activity in placental insulin receptor preparations.

It is accepted that the initial event of insulin action is the binding of insulin to its receptor. The insulin receptor has been proposed to be a glycoprotein complex consisting of two M_r 125 000 (α) and two M_r 94 000 (β) subunits linked together by disulfide bonds (Yip et al., 1980; Pilch & Czech, 1980; Jacobs et al., 1980; Massagué et al., 1980, 1981; Van Obberghen et al., 1981; Hedo et al., 1981). The regulation of cellular metabolism following the interaction of insulin with its receptor is achieved primarily by the phosphorylation and dephosphorylation of enzymes [reviewed by Cohen (1982); Denton et al., 1981]. Denton et al. (1981) proposed that activation of a membrane-associated insulin-dependent protein kinase may be an early, if not primary, event in metabolic regulation by insulin. Recent data have indicated that the β subunit of the insulin receptor can itself undergo an insulin-dependent phosphorylation in lymphocytes (Kasuga et al., 1982a) and hepatomas (Kasuga et al., 1982b) as well as in soluble, cell-free systems (Van Obberghen & Kowalski, 1982; Kasuga et al., 1982c). A tyrosine residue is the main target for this insulin-dependent phosphorylation in the cell-free system (Kasuga et al., 1982c). The demonstration that an insulin receptor tyrosine residue is a hormone-dependent protein kinase substrate parallels similar observations for a variety of other mitogenic polypeptide growth factors and their respective receptors. These include receptors for epidermal growth factor (EGF)[1] (Ushiro & Cohen, 1980), platelet-derived growth factor (PDGF) (Ek et al., 1982; Nishimura et al., 1982), and insulin-like growth factor 1 (IGF-1) (Paul Pilch, unpublished observations). In the case of the epidermal growth factor receptor, evidence was presented that the receptor is also the protein kinase that catalyzes a self-phosphorylation reaction (Cohen et al., 1980; Buhrow et al., 1982). In the present study we demonstrate that the β subunit of the insulin receptor is an insulin-dependent protein kinase capable of self-phosphorylation on a tyrosine residue.

Materials and Methods

Receptor Preparation. A partially purified human placental insulin receptor was prepared by a modification of the procedure of Williams & Turtle (1979). Placental microsomes were prepared and solubilized as described (Williams & Turtle, 1979) with the inclusion of 1 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride during homogenization of the

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[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor 1; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; AdoPP(NH)P, adenosine 5′-(β,γ-imidotriphosphate); IgG, immunoglobulin G.

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placenta. The soluble fraction was then chromatographed on Sephacryl S-400 (Pharmacia) and hydroxylapatite as described (Williams & Turtle, 1979). The insulin-binding fractions from the hydroxylapatite column were pooled and placed on a 1 × 10 cm DEAE-trisacryl (LKB) column and eluted with a 0.1–1.0 M NH_4Cl gradient in 50 mM sodium acetate, pH 6.3–0.1% Triton X-100 [see Siegel et al. (1981)]. The insulin-binding fractions from this column were pooled and dialyzed against 50 mM Hepes, pH 7.6, containing 0.1% Triton X-100. These preparations had a specific insulin-binding capacity of 50–150 pmol of insulin bound/mg of protein, which we estimate represents 3–5% pure receptor.

Phosphorylation Assay. Insulin receptor prepared as above (20–40 μg of protein) was incubated with or without insulin for 10 min at 23 °C, and then phosphorylation was initiated by the addition of [γ-^{32}P]ATP. Final concentrations were 3.5 × 10^{-7} M insulin, 1 mM Mg^{2+}, 50 μM [γ-^{32}P]ATP (5–15 μCi), 0.05–0.1% Triton X-100, and 50 mM Hepes, pH 7.6, in a final volume of 100 μL. The reactions were carried out for the desired time(s) and then terminated by the addition of electrophoresis sample buffer (final concentrations: 50 mM Tris, 50 mM dithiothreitol, 10% glycerol, 1% $NaDodSO_4$, pH 6.8) and 5 min at 95 °C or by the addition of unlabeled ATP to 1 mM (see Immunoprecipitation of the Insulin Receptor).

8-Azido[α-^{32}P]ATP Photolabeling. To the partially purified insulin receptor (30–50 μg) in the buffer indicated above was added 8-azido[α-^{32}P]ATP (0.5 μM, 4–11 μCi) in subdued light. After 8 min at 23 °C the incubation mixture containing the azido-ATP and insulin receptor preparation was directly illuminated from above with light from a focused 200-W mercury lamp (Oriel Corp.) for 15 s. In some preparations, adenosine 5′-(β,γ-imidotriphosphate) [AdoPP(NH)P] (Boehringer Mannheim) was present at a final concentration of 1 mM. Following photolysis, samples were either mixed with electrophoresis sample buffer (see above) for 5 min at 95 °C or subjected to immunoprecipitation as described below.

Immunoprecipitation of the Insulin Receptor. The partially purified insulin receptor preparation, untreated, phosphorylated, or photolabeled, was mixed in 100-μL portions (30–60 μg of protein) with 50 μL of a 1:50 dilution in 50 mM Hepes, pH 7.6, of monoclonal mouse anti-insulin receptor antibody (Kull et al., 1982) or with a similar amount of an unrelated monoclonal antibody. After 4–16 h at 4 °C, 25 μL of goat anti-mouse antibody (Cappel Corp.) was added, and incubation was continued for another 4–16 h. Finally 1.0 mL of 50 mM Hepes–0.1% Triton X-100, pH 7.6, was added, and the immunoprecipitate was centrifuged for 2 min at 15000*g*. This precipitate was washed 4 times and then dissolved in electrophoresis buffer as described above. In the case of untreated receptor preparations (Figure 3C), a phosphorylation assay as described above was carried out prior to electrophoresis in the presence of sodium dodecyl sulfate.

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was accomplished on 5% or 7.5% acrylamide gels as described (Laemmli, 1970). Autoradiography of Coomassie blue stained, dried gels was accomplished at −70 °C with Kodak X-Omat AR film and Cronex lightning plus enhancing screens. Protein concentrations were determined by the fluorescamine method (Bohlen et al., 1973).

Two-dimensional thin-layer electrophoresis was performed exactly as described by Hunter & Sefton (1980).

Monocomponent porcine insulin was a gift from Dr. R. Chance, Eli Lilly Co. Anti-insulin receptor antibody was a gift from Dr. S. Jacobs, Wellcome Research Laboratories. [γ-^{32}P]ATP was obtained from New England Nuclear Corp. and 8-azido[α-^{32}P]ATP was purchased from ICN Corp.

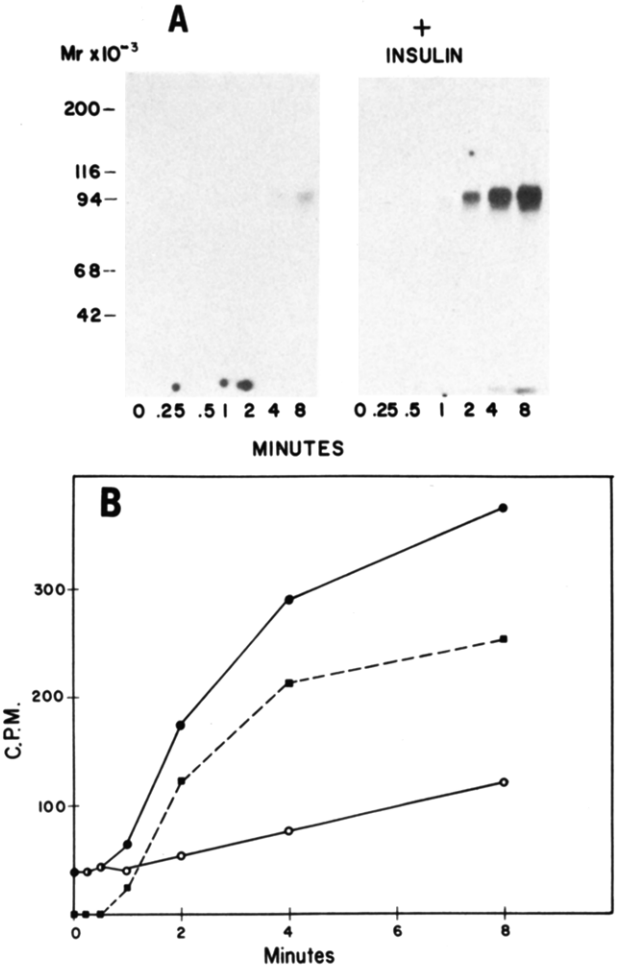

FIGURE 1: Time course of ^{32}P incorporation from [γ-^{32}P]ATP into an M_r 94000 component of human placental membranes. (A) Partially purified insulin receptor preparations were incubated with insulin (3.5 × 10^{-7} M) or not and then exposed to [γ-^{32}P]ATP for the times indicated. Phosphorylation was terminated by heating the protein to 95 °C for 5 min, and then electrophoresis on a 7.5% polyacrylamide gel was performed. Shown is an autoradiogram of a stained, dried gel. The position of molecular weight markers is shown on the left (myosin, M_r 200000; β-galactosidase, M_r 116000; phosphorylase *b*, M_r 94000; bovine albumin, M_r 68000; actin, M_r 42000). (B) The M_r 94000 region of the gel in panel A was excised, and the Cerenkov radiation in this region was determined in a scintillation counter. The open circles depict the values in the absence of insulin, the closed circles are the values in the presence of insulin, and the dotted line (squares) is the difference between the two.

Results

The time course of ^{32}P incorporation from [γ-^{32}P]ATP into a partially purified insulin receptor preparation is shown in Figure 1. Panel A is an autoradiogram after $NaDodSO_4$–polyacrylamide gel electrophoresis of the phosphorylation assay mixture. The only labeled band migrates at M_r 94000, the molecular weight of the β subunit of the insulin receptor, and insulin markedly stimulates incorporation of ^{32}P into this band. Incorporation of radioactivity in the M_r 94000 species was determined by measuring the Cerenkov radiation from the appropriate region of the dried gel. The results are plotted in panel B. The insulin-stimulated phosphorylation plateaus at 4–8 min (dotted line), and a 5–6-fold stimulation of ^{32}P incorporation due to insulin is evident at these times (closed circles vs. open circles).

Figure 2 illustrates the insulin dependency of the label incorporation into the M_r 94000 protein. Phosphorylation was allowed to proceed for 8 min after a 10-min preexposure to the insulin concentration indicated. Electrophoresis and au-

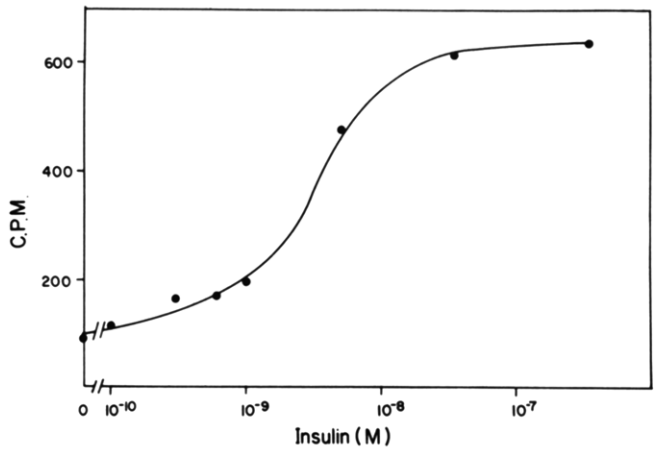

FIGURE 2: Insulin dependency of label incorporation into the M_r 94000 component of placental membranes. A partially purified insulin receptor preparation (see Materials and Methods) was exposed to the insulin concentration indicated for 10 min at 23 °C. [γ-^{32}P]ATP was then added and phosphorylation was allowed to proceed for 8 min. The reaction was then stopped, and subsequent steps of electrophoresis, autoradiography, and radioactivity determinations in the M_r 94000 region were carried out as described under Materials and Methods and in the legend to Figure 1.

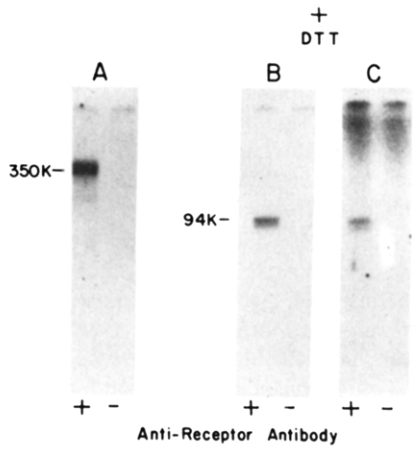

FIGURE 3: Immunoprecipitation of the insulin receptor. A phosphorylation assay was carried out in the presence of 3.5×10^{-7} M insulin for 8 min at 23 °C. Unlabeled ATP to 1 mM was then added, and immunoprecipitation with anti-receptor antibody or control antibody was performed as described under Materials and Methods. A portion of these immunoprecipitates were subjected to electrophoresis in the absence of a reducing agent on a 5% gel [acrylamide:*N*,*N*-methylenebis(acrylamide), 30:0.3] as depicted in panel A. The electrophoresis in panel B was performed on a 7.5% gel [acrylamide:*N*,*N*-methylenebis(acrylamide), 30:0.8] in the presence of 50 mM dithiothreitol. In panel C, the phosphorylation assay was carried out after the immunoprecipitation of the receptor. Depicted are autoradiographs of stained, dried gels.

toradiography confirmed that only the M_r 94000 band was phosphorylated. ^{32}P incorporation into this band was assessed by determining the Cerenkov radiation of the appropriate area of the dried gel. As can be seen in Figure 2, ^{32}P incorporation is saturable with respect to the insulin concentration and is half-maximal at $(2–3) \times 10^{-9}$ M insulin. This corresponds exactly to the K_d for [^{125}I]insulin binding to this preparation as determined by Scatchard analysis (data not shown).

The data in Figures 1 and 2 show only an insulin-dependent phosphorylation of an M_r 94000 band. We used immunoprecipitation by an anti-insulin receptor antibody to determine that this band is indeed a subunit of the insulin receptor. Figure 3 shows an autoradiogram of phosphorylated receptor preparations following electrophoresis. In panel A, electrophoresis of the phosphorylated, immunoprecipitated placental

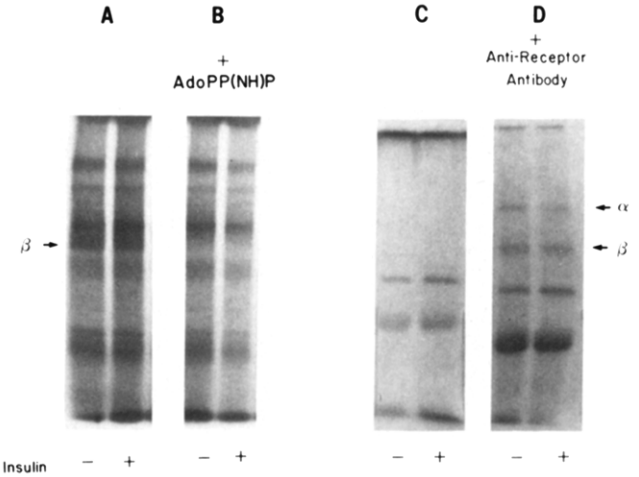

FIGURE 4: Photolabeling of the insulin receptor with 8-azido[α-^{32}P]ATP. The photoincorporation of 8-azido[α-^{32}P]ATP into a partially purified insulin receptor preparation in the presence (B) and absence (A) of adenosine 5′-(β,γ-imidotriphosphate) [AdoPP(NH)P] was accomplished as described under Materials and Methods. Insulin (3.5×10^{-7} M) was incubated with receptor for 10 min at 23 °C prior to photolysis where indicated (+). Photolabeled receptor was also immunoprecipitated with anti-insulin receptor antibody (D) or control antibody (C). Depicted are autoradiographs of stained, dried gels. The receptor subunits are denoted by α and β.

preparation was conducted in the absence of dithiothreitol. Panels B and C contained this reductant in the electrophoresis sample buffer. Specifically immunoprecipitated in panel A and panel B are phosphorylated bands of M_r 350000 and M_r 94000, respectively. These bands have previously been determined to be the intact, disulfide-linked insulin receptor (M_r 350000) and its β subunit by a variety of techniques (Yip et al., 1980; Pilch & Czech, 1980; Jacobs et al., 1980; Massaguē et al., 1980, 1981; Van Obberghen et al., 1981; Hedo et al., 1981). The phosphorylated species obtained in the absence of reductant was verified to be the intact $\alpha_2\beta_2$ receptor form (M_r 350000) and not a proteolytically altered receptor form (Massaguē et al., 1981) by comparison with the affinity-labeled insulin receptor (data not shown). These data, shown in Figure 3A,B, indicate that the β subunit (M_r 94000) of the intact insulin receptor is a substrate for an insulin-dependent protein kinase. In panel C, the phosphorylation assay was carried out after immunoprecipitation of an untreated, partially pure receptor preparation. This immunoprecipitate was also able to phosphorylate the β subunit, suggesting that the receptor itself is a protein kinase. Control antibody was unable to immunoprecipitate receptor in each of the above cases (panels A–C).

To further document that the receptor was itself a protein kinase, we photolabeled the partially purified placental preparation with 8-azido[α-^{32}P]ATP, an ATP analogue that can photoincorporate into ATP binding sites of proteins (Haley & Hoffman, 1974). As shown in panels A and B of Figure 4 this analogue, when photoactivated, labels a large number of bands. However, when an excess of the nonhydrolyzable ATP derivative adenosine 5′-(β,γ-imidotriphosphate) [AdoPP(NH)P] (Yount et al., 1971) is included in the incubation mixture (panel B) to block incorporation of 8-azido-ATP into ATP binding sites, incorporation of ^{32}P is markedly decreased only in the M_r 94000 protein (β). Furthermore, as shown in panel D, anti-insulin receptor antibody specifically immunoprecipitates azido[α-^{32}P]ATP linked to the M_r 94000 protein, indicating that this band is indeed the β subunit. The label incorporated into the α subunit of the receptor demonstrated in panel D is not specific for an ATP binding site because AdoPP(NH)P has no effect on this band (panel B).

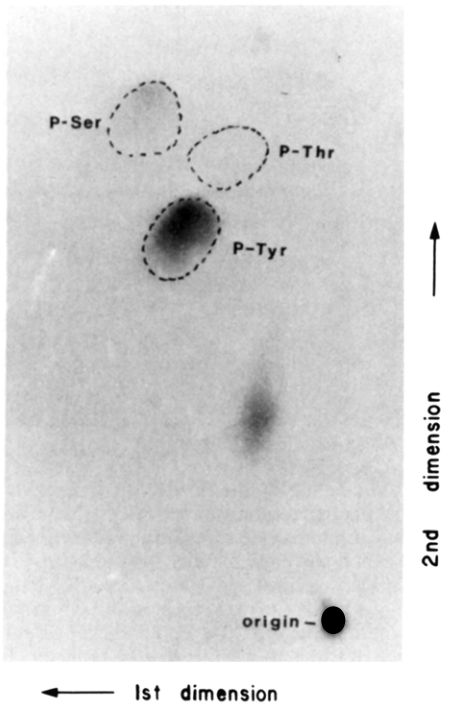

FIGURE 5: Two-dimensional thin-layer electrophoresis of phosphoamino acids. Immunoprecipitated, phosphorylated insulin receptor (see Materials and Methods) was hydrolyzed for 2 h at 110 °C in 6 N HCl. The hydrolysate was freeze-dried and applied to a 250-μm cellulose TLC plate. Electrophoresis conditions were exactly as described by Hunter & Sefton (1980). The dotted lines represent the ninhydrin-stained standards superimposed on an autoradiograph.

Control antibody does not precipitate either the α or the β subunit of the insulin receptor (Figure 4C). There is no apparent insulin effect on incorporation of 8-azido[α-^{32}P]ATP into the β subunit of the insulin receptor (parts A and D of Figure 4, minus vs. plus insulin).

It is evident in panels C and D of Figure 4 that there are labeled bands that migrate faster than the insulin receptor subunits. These bands have apparent molecular weights of 68 000 and 50 000 and comigrate with albumin and the heavy chain of IgG, respectively, as visualized by Coomassie blue staining of gels after $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). It is not clear why ^{32}P is incorporated into these proteins under the conditions of our immunoprecipitation, but it occurs for both control (panel C) and anti-receptor antibody (panel D). It seems most likely that this label incorporation occurs during the immunoprecipitation protocol because the M_r 68 000 and M_r 50 000 bands do not correspond to any bands in our partially pure receptor preparation, as determined by either Coomassie blue staining or silver staining (data not shown). Furthermore, these bands do not correspond to any band directly labeled by 8-azido[α-^{32}P]ATP, as shown in Figure 4A,B. Incorporation of ^{32}P into albumin and IgG is peculiar to the use of 8-azido[α-^{32}P]ATP since we encountered no such phenomenon when immunoprecipitating the insulin receptor in the presence of [γ-^{32}P]ATP (Figure 3).

Previous studies of receptor phosphorylation in a cell-free system from rat liver have indicated that the stimulated phosphorylation occurred mainly on a tyrosine residue (Kasuga et al., 1982c). We determined the site of phosphorylation in the placental insulin receptor by thin-layer electrophoresis of amino acids following hydrolysis of the phosphorylated receptor. As shown in Figure 5, a trace of phosphoserine is evident but, as in prior studies, phosphotyrosine is the principal phosphoamino acid produced by insulin treatment of receptor preparations in the presence of [γ-^{32}P]ATP.

Discussion

We present two lines of evidence in this report that are consistent with the hypothesis that the β subunit of the insulin receptor is a protein kinase. The first line of evidence is that anti-insulin receptor antibody can be used to immunoprecipitate protein kinase activity (Figure 3C). This does not identify which subunit, α or β, is the active one. To address this question, we photoincorporated 8-azido[α-^{32}P]ATP into the partially purified receptor preparation (Figure 4). This compound should bind in the dark to the ATP binding site of the kinase and become covalently incorporated upon photolysis (Haley & Hoffman, 1974). In fact, we see numerous protein bands to which the ATP analogue has become coupled, including both subunits of the insulin receptor. This is not surprising since the negatively charged 8-azido-ATP is free to associate with all positively charged protein groups as well as ATP binding sites. Under the conditions of the experiment shown in Figure 4, a certain amount of kinase activity and possibly ATPase activity are also likely to occur, thus complicating analysis. Nevertheless, when excess AdoPP(NH)P is included to block binding of 8-azido-ATP to the ATP binding site(s), inhibition of label incorporation occurs only for the M_r 94 000 band (Figure 4B), the β subunit of the insulin receptor as demonstrated by immunoprecipitation (Figure 4D). Taken together with the data showing the acute insulin sensitivity of the kinase reaction (Figures 1 and 2), it is highly likely that the β subunit of the insulin receptor is an insulin-dependent, tyrosine-preferring (Figure 5) protein kinase.

It is not clear whether the insulin receptor is itself a primary target for the insulin-dependent phosphorylation or whether this phosphorylation is a fortuitous reflection of another cellular target. Such a target could be an enzyme important in the regulation of metabolism by phosphorylation and dephosphorylation (Denton et al., 1981). The phosphorylation of tyrosine is a very rare event and phosphotyrosine comprises only 0.05% of the phosphoamino acids in proteins (Sefton et al., 1980). By comparison with the control of metabolism by cAMP-dependent protein kinase activity, it would appear that the insulin-dependent, receptor-mediated protein kinase activity is insufficient to directly regulate metabolism. However, one cannot rule out a cascade mechanism that would amplify this insulin-dependent signal to levels consistent with metabolic control. Another possible role of the tyrosine-specific phosphorylation is regulation of growth control. Certain virally transformed cells have greatly enhanced phosphotyrosine levels due to the activity of the viral protein kinase, pp 60v-src (Sefton et al., 1980). Moreover, several mitogenic peptide growth factors are capable of mediating tyrosine-specific protein kinase activity. These include EGF (Cohen et al., 1980), PDGF (Ek et al., 1982; Nishimura et al., 1982), and IGF-1 (Paul Pilch, unpublished observations). Insulin is also a general requirement for cell growth in culture. Thus, the link between cell growth and the tyrosine-specific phosphorylations stimulated by the interaction of these various polypeptides with their respective receptors is an intriguing one.

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Registry No. ATP, 56-65-5; insulin, 9004-10-8; protein kinase, 9026-43-1.

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Comparison of Protein and Deoxyribonucleic Acid Backbone Structures in fd and Pf1 Bacteriophages†

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ABSTRACT: The conformations of the protein and nucleic acid backbones in the filamentous viruses fd and Pf1 are characterized by one- and two-dimensional solid-state NMR experiments on oriented virus solutions. Striking differences are observed between fd and Pf1 in both their protein and DNA structures. The coat proteins of fd and Pf1 are almost entirely α helical and in both viruses most of the helix is oriented parallel to the filament axis. fd coat protein is one stretch of α helix that is slightly slued about the filament axis. In Pf1 coat protein two distinct sections of α helix are present, the smaller of which is tilted with respect to the filament axis by about 20°. The DNA backbone structure of fd is completely disordered. By contrast, the DNA backbone of Pf1 is uniformly oriented such that all of the phosphodiester groups have the O–P–O plane of the nonesterified oxygens approximately perpendicular to the filament axis.

The filamentous bacteriophages are nucleoprotein complexes of apparently simple design (Marvin & Hohn, 1969). Approximately ten of these viruses have been isolated. They are all filaments with a single-stranded circle of DNA surrounded by several thousand copies of a small protein. The coat protein subunits are arranged symmetrically in a helical array. Two classes of viral structures have been identified on the basis of X-ray diffraction patterns from oriented fibers of the viruses. The class I viruses (Marvin et al., 1974a) such as fd, M13, and If1 have distinctly different symmetry than the class II viruses (Marvin et al., 1974b) Pf1 and Xf.

This paper presents structural data for the class I virus fd and the class II virus Pf1 that directly address some of the most important questions concerning differences between these viruses (Day & Wiseman, 1978; Marvin et al., 1974a,b). The 1:1 ratio of nucleotides to coat protein subunits for Pf1 suggests specific protein–nucleic acid interactions and limits the possible DNA conformations. By contrast, this ratio is 2.3:1 for fd, which not only is much larger than that for Pf1 but also is significantly nonintegral, implying that the protein–nucleic acid interactions are nonspecific. There is general agreement that the coat proteins of both fd and Pf1 have a very high per-

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