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Protein Kinase Activity of the Insulin Receptor in Human Circulating and Cultured Mononuclear Cells

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SUMMARY - In lectin-purified receptor preparations from human monocyte-like cell (U-937), insulin (10^{-7}M) stimulated phosphorylation of the 95,000 dalton subunit of its own receptor. In addition, insulin stimulated phosphorylation of exogenously added substrates like casein, (T,G)-A--L, and histones. Phosphorylation of the synthetic peptide (T,G)-A--L indicates the presence of at least one insulin-dependent tyrosine kinase in these cell extracts. Insulin receptor preparations from freshly isolated human mononuclear blood cells were also shown to possess insulin-dependent casein and (T,G)-A--L kinase activity. Phosphorylations in these systems are specific for insulin and dependent on insulin concentration. A simple and rapid method is described that is relevant for clinical investigations of early postbinding events.

The binding of insulin to its cell surface receptor stimulates a protein kinase that leads to phosphorylation of at least the 95K subunit of the receptor (1-4). The tight association of the kinase activity with the insulin receptor suggests that the receptor is both a protein kinase and a substrate for the kinase. While the physiological significance of insulin receptor phosphorylation is unknown, it is clear that this is a very early event following binding and a possible site of pathology in altered states of insulin action.

The circulating monocyte is a useful cell for studies of altered states of insulin binding in people (5,6). Further, the U-937 monocyte-like cell line is an excellent in vitro model of the circulating monocyte (7).

Abbreviations

HEPES, 4-(2-hydroxyethyl) - 1-piperazineethanesulfonic acid; (T,G)-A--L, (L-tyr, L-glu) - poly (D,L-ala)-poly (L-lys); LHRH, luteinizing-hormone releasing hormone, hCG, human chorionic gonadotropin.

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We show in this report that insulin-stimulated phosphorylation of exogenously added substrates occurs with solubilized receptor preparations from both the cultured cells and freshly isolated peripheral blood cells. Additionally, insulin stimulates phosphorylation of the β subunit of the insulin receptor of the human monocyte-like cell line. Thus these cells provide us with a model for early postbinding steps following insulin's interaction with its receptor.

METHODS

Cultured human monocyte-like cells (U-937) (7) or freshly isolated normal human mononuclear cells (8) (from 100 ml blood) were used as the source of kinase. Cells were disrupted by sonication in buffer containing 0.25 M sucrose, 50 mM HEPES, aprotinin (1 T.I.U./ml) and 1 mM PMSF, pH 7.6. The cellular suspension was centrifuged at 550 xg, for 5 min. at 4°C and the supernatant further spun at 190,000 xg, for 120 min. The pellet was solubilized in 50 mM HEPES, pH 7.6, 1% Triton-X, and 1 mM PMSF at 4°C for 30 min. and then centrifuged at 120,000 xg, for 60 min. The supernatant was applied to a wheat germ agglutinin column as previously described (6). The solution eluted from the column was denoted "lectin-purified insulin receptors". Receptor preparations were incubated with insulin or other hormones (final concentration 10^{-7}M) for 60 min. at 4°C. Exogenous substrates (casein, histone H2b, (T,G)-A--L; final concentration 2.5 mg/ml) and Mg Cl_2 (final concentration 20 mM) were added, tubes were incubated at 22°C for 10 min. and phosphorylation was begun with addition of a solution containing [γ-^{32}P]ATP (0.45 mM, 2.2Ci/mmol). Reaction was terminated by spotting 75 µl aliquots on filter papers and placing them in a bath with 10% v/v TCA (with 10 mM Sodium pyrophosphate). After extensive washing the TCAprecipitable material was counted in a liquid scintillation counter. The amount of kinase catalyzing incorporation of 1 pmole of phosphate into 1 mg of exogenous substrate in 10 min. was equal to one unit of activity. All materials and reagents used were previously described (2).

RESULTS

U-937 cells were homogenized, the particulate fraction solubilized and applied to a wheat-germ column. This lectin-purified insulin receptor preparation was then incubated in the presence or absence of insulin with a reaction mixture containing [γ-^{32}P]ATP. The phosphoproteins were identified by immunoprecipitation followed by NaDodSO$_4$-polyacrylamide gel electrophoresis and autoradiography (1). Incubation of the receptor preparations with 10^{-7}M insulin followed by immunoprecipitation with serum containing anti-insulin receptor antibody resulted in identification of a 95,000-dalton band (lane D) which has been previously identified as the β subunit of the insulin receptor (9). In contrast, no difference

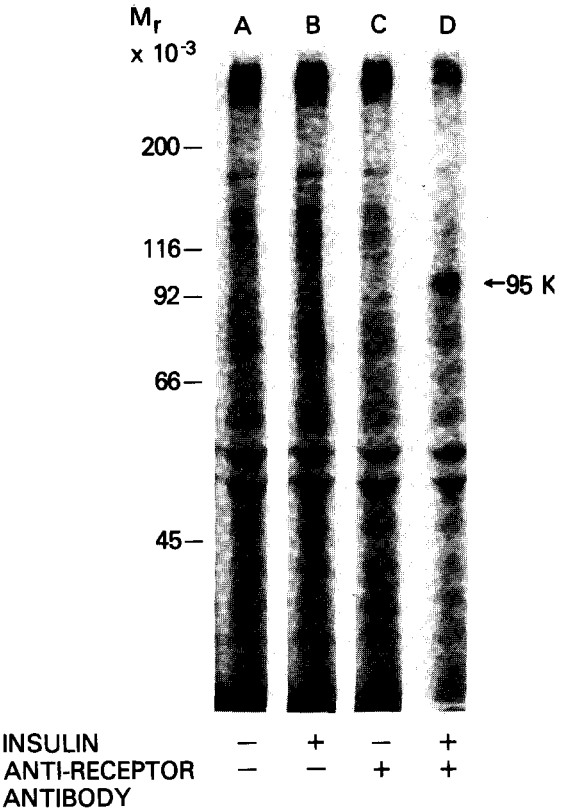

Fig. 1. Phosphorylation of insulin receptor from human monocyte-like cells. Autoradiogram shows ^{32}P labeling of the insulin receptor. Receptors were incubated with or without insulin (10^{-7}M) at 4°C for 60 min.prior to phosphorylation. Phosphorylation of lectin-purified receptor preparations was carried out as previously described (2). Lanes A and C show immunoprecipitates from cell extracts incubated in absence of insulin, lanes B and D in presence of insulin. Control serum was used for precipitation in lanes A and B, while anti-receptor antibody was used in lanes C and D. Phosphorylation of the Mr 95,000 subunit was stimulated specifically in lane D.

in pattern of phosphorylated bands was seen when normal serum was used for immunoprecipitation (Fig. 1, lanes A and B). We have previously shown that an insulin-dependent tyrosine kinase which is tightly associated with the receptor itself phosphorylates the insulin receptor as well as casein and histones (4). We therefore used exogenous substrates to further characterize the receptor kinase of the monocyte-like cell line. Figure 2 shows the effect of insulin (10^{-7}M) on phosphorylation of several exogenous substrates. Insulin clearly stimulated phosphorylation of casein, histone H2b, and the synthetic peptide, (T,G)-A--L, in a con-

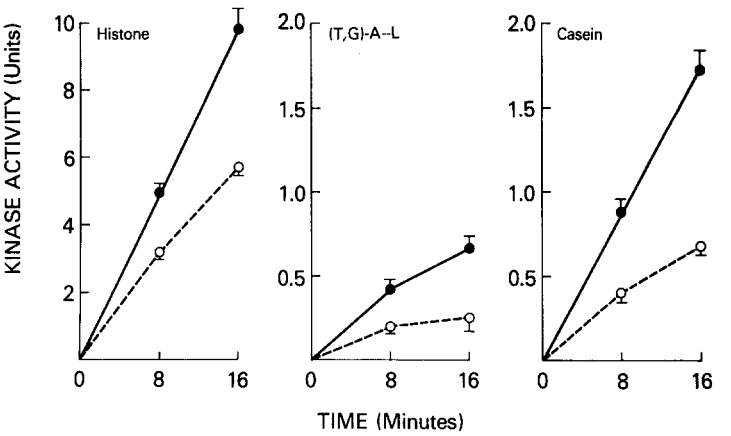

Fig. 2. Insulin-stimulated phosphorylation of exogenous substrates by U-937 extracts. In the experiments shown lectin-purified insulin receptor preparations from U-937 cells were incubated with (•) or without (o) insulin (10^{-7}M) for 60 min. at 4°C. Exogenous substrates (histone H2b, (T,G)-A--L, casein) were then added and phosphorylation initiated 10 min. later at 22°C. Data shown represent means ± standard error of three experiments.

centration- and time-dependent fashion. Optimal insulin-stimulated casein phosphorylation occurred at insulin concentration of 10^{-7}M (data not shown) as previously reported in other cell types (2).

Since tyrosine residues are the only ones available for phosphorylation in (T,G)-A--L, our results indicate the presence of at least one insulin-dependent tyrosine kinase in the U-937 receptor preparations. This was confirmed by phosphoamino acid analysis of the products of casein phosphorylation. The amount of p-tyrosine increased 5-6 fold but there was no change in the amount of p-serine/p-threonine after incubation with insulin.

To demonstrate that the observed stimulation of phosphorylation is specific for insulin, we assessed the effect of EGF, another growth factor capable of stimulating tyrosine kinase. In contrast to the insulin effect, no stimulation of (T,G)-A--L phosphorylation was seen with EGF (10^{-6} or 10^{-7}M) at any time up to 16 minutes. In summary, in the U-937 cells the insulin-dependent receptor and exogenous substrate phosphorylation appears to be carried out by the same enzyme. We also wished to probe for the presence of the receptor kinase in freshly isolated mononuclear blood cells by measuring the insulin-stimulated casein phosphorylation. When lectin-purified

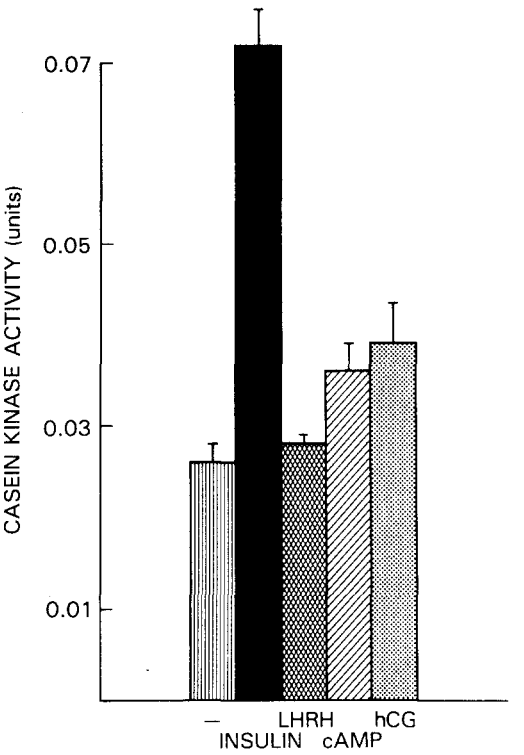

Fig. 3. Specificity of insulin-stimulated phosphorylation of casein by insulin receptor preparations from freshly isolated human mononuclear blood cells. Casein phosphorylation was carried out in absence or presence of the various hormones (final concentration 10^{-7}M) as described in the Methods.

receptor preparations from mononuclear cell fraction of normal blood were incubated with insulin, phosphorylation of both (T,G)-A--L and casein were markedly stimulated (two- or three-fold). This effect was dose dependent with maximal stimulation occurring at insulin concentration of 10^{-7}M (data not shown). Further, the insulin effect was specific (Fig. 3). cAMP as well as LHRH or hCG had no effect on phosphorylation of casein.

DISCUSSION

Using lectin-purified insulin receptor preparations from human monocyte-like cells we show in this report that insulin stimulates phosphorylation of the β subunit of its receptor. Further, insulin stimulates phosphorylation of exogenously added substrates, such as casein, (T,G)-A--L, and histone H2b by receptor preparations from both cultured and freshly isolated human

mononuclear cells. We have reported previously that the insulin-stimulated receptor and casein kinase activities are tightly associated and both reactions are catalyzed presumably by a single enzyme (4). We conclude that the insulin-stimulated casein phosphorylation measured in freshly isolated blood cells represents the insulin receptor kinase activity. This conclusion is supported by the finding of parallel receptor and casein phosphorylation in the U-937 cells. The insulin receptor of the U-937 cell line has been characterized and possesses many features similar to that of a monocyte (7).

The system described here has several features suitable for clinical investigations. The amount of blood used (100 ml) for the receptor preparation is equal to that used routinely to study insulin binding in various physiologic and disease states (5). The method of insulin-stimulated casein phosphorylation is simple and rapid, allowing screening of many patients without the necessity of laborious studies of phosphorylation of the receptor itself. In addition, the lectin-purified receptor preparations can be stored frozen for several weeks. There is, of course, a potential for use of other blood cell types such as B-lymphocytes (Y.Z., unpublished observations) or erythrocytes (10), in clinical studies of insulin stimulated protein kinase. Investigations of postbinding events such as the receptor phosphorylation could serve as a key to elucidate defects in insulin action in patients with insulin resistance but normal receptor binding.

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