Insulin-like Growth Factor I and Insulin Rapidly Increase Casein Kinase II Activity in BALB/c 3T3 Fibroblasts*

(Received for publication, April 5, 1988)

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We have tested whether growth factors added to serum-deprived BALB/c 3T3 fibroblasts alter the casein kinase II activity measured in cell extracts. A rapid phosphocellulose chromatography method was developed that provides a 40-fold partial purification of casein kinase II activity associated with the specific substrate peptide Arg-Arg-Glu-Glu-Thr-Glu-Glu-Glu. Using this technique, kinase activity is stimulated 1.6–2.6-fold when isolated from fibroblasts treated with insulin or insulin-like growth factor I (IGF-I). The activated kinase activity exhibits the specific properties of casein kinase II such as the ability to utilize [γ-32P]GTP as phosphate donor and marked inhibition by low concentrations of heparin. Activation of casein kinase II appears specific for these hormones because epidermal growth factor and platelet-derived growth factor have no effect on the kinase activity when added to fibroblasts under conditions where they markedly stimulate [3H]thymidine incorporation into DNA. Increases of casein kinase II activity by insulin and IGF-I were detected within 1 min of their addition to cell cultures. IGF-I is more potent in stimulating casein kinase II than insulin in mouse fibroblasts. These results demonstrate that casein kinase II is a selective target for insulin and IGF-I action in BALB/c fibroblasts, consistent with the hypothesis that this kinase plays a role in cellular signaling by these hormones.

Insulin and insulin-like growth factor I (IGF-I) are structurally related peptides that modulate a large number of similar cellular processes. Included among their major biological effects are activation of nutrient transport and receptor-mediated ligand uptake systems, modulation of metabolic enzyme activities, transcriptional regulation of specific genes, and stimulation of cell proliferation (for reviews see Refs. 1–4). A common feature of several of the target enzymes that have been well characterized is that regulation involves phosphorylations or dephosphorylations of serine or threonine residues, or both. Although the insulin-mediated activation of several important enzymes such as glycogen synthase (5–7) and pyruvate dehydrogenase (8) is associated with dephosphorylation, quantitatively the predominant effect of insulin in target cells is to increase serine/threonine phosphorylation of numerous proteins (9). Known intracellular proteins that exhibit increased phosphorylation in response to insulin include the S6 ribosomal protein (10), ATP citrate lyase (11, 12), and acetyl-CoA carboxylase (13, 14). In the case of these proteins, however, the relationship between altered phosphorylation sites and changes in function has not been established.

The serine/threonine protein kinases that mediate the increased protein phosphorylation due to insulin are poorly understood at present. One or more specific S6 protein kinases have been shown to be activated by a number of growth factors in addition to insulin (15). Purification of an S6 kinase has recently been achieved (16–18), but no information is yet available on regulatory mechanisms. We recently found a novel membrane-bound protein kinase activity that is activated by insulin in isolated adipocytes (19). An insulin-stimulated cytosolic protein kinase with the ability to catalyze phosphorylation of ATP citrate lyase and Leu-Arg-Arg-Ala-Leu-Gly (Kemptide) in vitro has also been recently identified (20). The degree to which these newly discovered insulin-sensitive serine/threonine kinases contribute to the augmented cellular phosphorylations due to insulin action remains to be clarified.

Acetyl-CoA carboxylase and phosphatase inhibitor 2 are phosphorylated by casein kinase II in vitro (13, 21). Phosphorylation of both proteins is stimulated by insulin in adipocytes (13, 22). It was therefore of interest to investigate whether casein kinase II might be regulated by hormones, and we investigated the activity of this enzyme in extracts of fibroblasts after stimulation by a number of growth factors. The data presented in this report show activation of a kinase with characteristics similar to casein kinase II in response to insulin and IGF-I in mouse fibroblasts.

MATERIALS AND METHODS

Cell Cultures—BALB/c 3T3 fibroblasts were kindly provided by Dr. Roger Davis. They were grown in Dulbecco’s modified Eagle’s medium and split twice weekly before reaching confluence.

Purification of Casein Kinase II on Phosphocellulose—The cells were washed twice with 5 ml of ice-cold lysis buffer (15 mM Hepes, pH 7.4, 8 mM nitrophenyl phosphate, 8 mM 5-β-glycerophosphate, 4 mM sodium vanadate, 4 mM EDTA, 60 mM NaF, 60 mM NaCl). Excess buffer was aspirated off the plates, and the cells were removed from the plates with a rubber policeman. The cells were immediately lysed with 0.25% Triton X-100, and the extracts were clarified by centrifugation at 15,000 x g for 5 min. Total protein concentrations in the extracts were determined by the Bio-Rad Coomasie Blue assay using bovine serum albumin as standard. Approximately 300 μl of packed volume phosphocellulose (Bio-Rad) was added to the supernatants, and the samples were incubated for 1 h on an end-over-end agitator at 6 °C. The phosphocellulose was then washed 6 times with 1 ml of assay buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 0.5 mM sodium vanadate, 1 mM dithiothreitol). Elution was performed by 10 min of incubation with 1 ml of assay buffer containing 12 mM NaCl and 0.1% bovine serum albumin, and the eluate was concentrated by centrifugation in Centricon 30 microconcentrators (Amicon) to 45 μl. Desalting was accomplished by adding 600 μl of assay buffer to the concentrate and centrifuging the microconcentrators again, and this...
step was repeated once. All these procedures were performed at 0-6 °C.

**Kinase Assays**—The peptide substrate for casein kinase II was synthesized on an Applied Biosystems Inc. peptide synthesizer and purified by high pressure liquid chromatography. Assays were performed in 5-μl aliquots of the concentrates with 100 μM \([\gamma-\text{P}]\text{GTP}\) (5 μCi) for 10 min at 30 °C. The reactions were stopped by adding 20 μl of 0.2 M HCl, 5 mM ATP, and 1% bovine serum albumin. The protein was precipitated by the addition of 10 μl of 12.5% trichloroacetic acid and centrifuging for 5 min at 15,000 x g. The radioactivity incorporated into the peptides was determined by spotting the extracts after transfer to assay buffer in Centricon 30 microcentrifugators. No significant difference in yield was observed when insulin-treated cells were used.

**Hormone Stimulation of Cells and Thymidine Uptake**—EGF was from Sigma, human IGF-I was from Amgen, and human insulin was a generous gift from Dr. Ronald Chance at Lilly. The BALB/c 3T3 cells were seeded in 15-cm plastic tissue culture plates and were transferred to serum-free medium containing 2.5 μg/ml transferrin the day before reaching confluence. Hormone stimulation was performed 2 days later by aspirating the medium and adding 1 ml of medium containing the hormone and 20 μM Hepes and incubating at 36 °C for the indicated times. The [3H]thymidine uptake was determined in parallel cultures in 24-well tissue culture vessels that had been similarly serum-starved. After removing the culture medium, 50 μl of the hormone-containing medium was added to the plates, and, after 1 h, an additional 1 ml of medium containing transferrin and [3H]thymidine (2 μCi/ml, 20 μM) was added. After incubating for 6 h, incorporation was determined by lysing the cells in 1% sodium dodecyl sulfate, precipitating with trichloroacetic acid on Whatman 541 filters, and counting in a β-counter.

**RESULTS**

In order to examine the possible modulation of casein kinase II activity in response to hormone treatment, we developed experimental conditions that would allow minimal dephosphorylation to occur during the procedures of the kinase assay. One difficulty in measuring the activity of this kinase in cell extracts is that it is inhibited by the phosphatase inhibitors that are commonly added to preserve the phosphorylation state of proteins. For example, casein kinase II is inhibited by sodium vanadate in excess of 0.5 mM, and 50 mM NaF completely inhibits the activity of the enzyme. To overcome this problem, a procedure was devised that involves preparing cell extracts in the presence of phosphatase inhibitors and partially purifying casein kinase II on phosphocellulose, which removes the inhibitors. The kinase activity in the partially purified preparation was then measured utilizing a substrate peptide Arg-Arg-Glu-Glu-Thr-Glu-Glu-Glu-Thr that has been previously described to be specific for casein kinase II (24). A highly specific property of casein kinase II is the ability to utilize \([\gamma-\text{P}]\text{GTP}\) as phosphate donor (25). We therefore used this nucleotide in all assays.

Table I shows that addition of either insulin or IGF-I to BALB/c fibroblasts increases the apparent casein kinase II activity in the partially purified preparations of cell extracts. A similar increase was also seen when the combination of insulin, IGF-I, EGF, and platelet-derived growth factor (PDGF) was added to the cells. In contrast, addition of EGF or PDGF to BALB/c fibroblasts results in no detectable increase in casein kinase II activity. To verify that the added EGF and PDGF are biologically active in these experiments, parallel cell cultures were incubated with [3H]thymidine, and the effects of these growth factors on its incorporation were determined. As shown in Table I, both EGF and PDGF induced marked increases in thymidine uptake, confirming the biological potency of the preparations of these growth factors under the conditions of these experiments.

The hormone-dependent kinase activity measured in the experiments of Table I shares two characteristic features of authentic casein kinase II phosphorylation of the casein kinase II substrate peptide and utilization of \([\gamma-\text{P}]\text{GTP}\). Another specific property of casein kinase II is its high sensitivity to inhibition by low concentrations of heparin. Fig. 1 shows
that both the control and hormone-activated kinase activities measured under our experimental conditions are indeed abolished by 250 ng/ml heparin, and half-maximal inhibition of the kinase activity is observed at 50 ng/ml heparin. This is consistent with the extreme sensitivity of authentic casein kinase II to inhibition by heparin (26).

The time course of hormone action on casein kinase II activity appears to be rapid. In Fig. 2 is shown the time course of activation of casein kinase II activity in response to IGF-I. An effect was detectable after only 1 min, and the maximum response was apparent after 15 min. The activity of casein kinase II thereafter declined, decreasing to basal values 2½ h after exposure to IGF-I (Fig. 2) or insulin (not illustrated).

The dose-response relationship between casein kinase II activity concentrations of insulin or IGF-I added to BALB/c fibroblasts was determined. Fig. 3, panel B, shows that the half-maximal response to IGF-I occurred at about 1 nM. The stimulatory effect of insulin was half-maximal at about 100 nM (panel A), indicating a much less sensitive response compared to that of IGF-I. However, the magnitudes of the responses to the two peptides were similar (Table I, Fig. 3). Comparison of the dose-response relationship of insulin and IGF-I to kinase activation as a percent of the response observed at the maximum tested concentrations of the hormones is illustrated in Fig. 3, panel C, and confirms that insulin is approximately 100-fold less potent than IGF-I under our experimental conditions.

DISCUSSION

The data presented in this report demonstrate that addition of insulin or IGF-I to BALB/c fibroblasts results in a rapid stimulation of a kinase activity that catalyzes phosphorylation of the casein kinase II substrate peptide. Although the results do not unequivocally establish that casein kinase II itself is activated by these bioactive peptides, this possibility seems likely. The highly acidic phosphorylation substrate used in this study corresponds to the unique substrate specificity of casein kinase II and is not phosphorylated in the presence of numerous other known protein kinases (24). Casein kinase I also exhibits specificity for substrates with highly acidic environments but does not catalyze phosphorylation of threonine which is present within the casein kinase II substrate (24). In addition, our experimental protocols utilized a partial purification step involving phosphocellulose chromatography known to be effective in isolating casein kinase II (25). Furthermore, casein kinase II is unique among serine/threonine kinases in that it is able to utilize GTP as well as ATP as phosphate donor, similar to the regulated kinase we report here. Finally, the hormone-activated kinase we observe is extremely sensitive to inhibition by low concentrations of heparin as is authentic casein kinase II (Fig. 1).

Recently, a protein kinase activity which catalyzes phosphorylation of the low density lipoprotein receptor was found to phosphorylate the casein kinase II substrate with low efficiency (27). This kinase differs from casein kinase II by its apparent molecular weight and by its catalytic properties. We cannot exclude the possibility at present that the insulin- and IGF-I-sensitive kinase described here represents this receptor kinase or an unknown protein kinase with phosphate donor and substrate specificity similar to casein kinase II.

A striking observation reported here is the lack of effect of EGF and PDGF on the casein kinase II-like activity in the BALB/c 3T3 fibroblasts under conditions where insulin and IGF-I exhibit marked effects (Table I). Conversely, EGF and PDGF enhanced 3Hthymidine incorporation into DNA 20–35-fold in the cells while insulin and IGF-I had little or no effect. Some bioactions of EGF, PDGF, insulin, and IGF-I, such as regulation of glycogen synthesis and intracellular pH, appear to be similar, but differences in their effects have been noted (30, 31). We find that under the conditions reported here, EGF and PDGF clearly stimulate incorporation of 3Hthymidine in the BALB/c cells, whereas insulin and IGF-I do not (Table I). Also, while phosphatidylinositol turnover is modulated by PDGF in certain cell types, insulin and IGF-I are ineffective (32). The results presented here reinforce the concept that some contrasting effects are initiated by the insulin and IGF-I receptor tyrosine kinases versus the receptor kinases for EGF and PDGF.

The finding that IGF-I is more potent in activating casein kinase II-like activity than insulin in BALB/c fibroblasts (Fig. 3) suggests the effects of both ligands are mediated through the IGF-I receptor system in these cells. This is not surprising because fibroblasts possess greater numbers of IGF-I receptors than insulin receptors (33). Furthermore, effects of high concentrations of insulin on human fibroblast proliferation have been shown to be mediated through the IGF-I receptor using specific receptor-blocking antibodies (34). However, the insulin and IGF-I receptors appear to mediate similar biological responses (e.g. hexose and amino acid transport activation) in tissues where they are present in reasonable abundance (35). While this paper was in the review process, Sommecorn et al. (36) reported that low concentrations of insulin enhance casein kinase II activity in differentiated 3T3-L1 cells. Taken together, those results and the present report indicate that receptors for both insulin and IGF-I are capable of mediating this response.

Casein kinase II contains two subunit types denoted as α
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and β which are associated in an α2-β complex (for reviews see Refs. 37 and 38). The α-subunit contains the catalytic site, and the β-subunit is phosphorylated in the autophosphorylation reaction (25, 39). The enzyme forms aggregates under certain ionic conditions (40). These structural characteristics suggest the presence of interesting yet incompletely understood regulatory mechanisms.

A number of target proteins for insulin action contain casein kinase II phosphorylation sites. These include glycogen synthase (41), phosphatase inhibitor II (21), acetyl-CoA carboxylase (3), and the IGF-II/mannose 6-phosphate receptor (42). Insulin has been shown to increase the phosphorylation of phosphatase inhibitor II (22) and acetyl-CoA carboxylase (13, 14) in 32P-labeled adipocytes. In the latter case, the site of phosphorylation is located on a sequence that is highly acidic and phosphorylated by casein kinase II in vitro, as expected. These data are consistent with the hypothesis that enhancement of casein kinase II activity by insulin is involved in mediating some protein phosphorylation alterations caused by these agents. However, increased phosphorylation of the IGF-II receptor and glycogen synthase would appear to oppose the known actions of insulin on these proteins (5–7, 43). These ambiguities need to be clarified in future work. Nonetheless, the rapid activation of casein kinase II suggests an important role of this enzyme in the biological effects of insulin.

Acknowledgments—The substrate peptide for casein kinase II was the kind gift of Dr. Lynn Heasley. The expert typing of Mary Halley and Karen Donahue is greatly appreciated.

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