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SUBSTRATE SPECIFICITIES OF INSULIN AND EPIDERMAL GROWTH FACTOR RECEPTOR KINASES

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SUMMARY: The abilities of insulin and EGF stimulated protein kinases to phosphorylate a series of exogenous substrates were compared using wheat germ lectin purified preparations of solubilized rat liver membranes. Three different kinds of substrates were found: substrates phosphorylated primarily by insulin stimulated kinase, substrates phosphorylated primarily by EGF stimulated kinase and substrates phosphorylated by both kinases to a similar extent. These results indicate that the insulin and the EGF receptor kinase have different, but overlapping, substrate specificities. In vivo, phosphorylation of cellular proteins by various hormone receptor kinases may be part of the signal transmission process for actions of the hormones. Different substrate specificities of kinases of different hormone receptors may therefore represent an important mechanism to preserve the specificity of the hormonal signal at the post receptor level.

Upon binding to their receptors, a number of hormones, including insulin and epidermal growth factor (EGF), activate kinases that phosphorylate tyrosine residues within the receptor itself (autophosphorylation) as well as within protein substrates (1-3). Protein kinase activities appear to be intrinsic to the receptors (4-6), and tyrosine phosphorylation of one or more cellular proteins by these receptors may be important for the signal transmission between hormone binding and some, or all, hormone actions. Kinases linked to receptors for different hormones could phosphorylate the same cellular protein(s) and/or phosphorylate proteins that are specific substrates for the respective hormone-stimulated kinase.

Substrate specificity could be an important mechanism to preserve the specificity of the hormonal signal at the postreceptor level. On the other hand, some hormones that stimulate tyrosine kinases elicit a number of common cellular

ABBREVIATIONS: EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetic acid; Glu4:Tyr1, synthetic polymer with a 4:1 ratio of glutamic acid residues and tyrosine residues; $NaDodSO_4$, sodium dodecyl sulfate.

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responses (7,8), possibly reflecting a common mechanism by which the hormonal signals are processed. Using wheat germ lectin-purified preparations from human placenta membranes, Pike et al. (9) have previously found similar, but not identical, characteristics of the insulin- and EGF stimulated kinases with respect to divalent metal ion requirements, nucleotide specificities and substrate specificities. To further examine the substrate specificities of insulin and EGF receptor kinase, we compared the abilites of these kinases to phosphorylate various exogenous substrates.

MATERIALS AND METHODS

Materials: Porcine monocomponent insulin and [^{125}I-Tyr^{A14}]monoiodoinsulin were generously supplied by Drs. Ronald Chance and Bruce Frank of the Eli Lilly and Company. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. Angiotensin II and Val^{5}-angiotensin II were from Bachem (Torrence, CA). The peptide with the sequence Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, a sequence related to the sequence surrounding the site of phosphorylation in the transforming protein kinase of the Rous sarcoma virus, was purchased from Peninsula (Belmont, CA). It will be referred to as src-related peptide. Epidermal growth factor, histone 2b, casein, and a synthetic polymer with a 4:1 ratio of glutamic acid residues to tyrosine residues were purchased from Sigma. The polymer will be referred to as Glu4:Tyr1. Materials for sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Biorad.

Preparation of Wheat Germ Lectin Purified Receptors: Livers of male Sprague Dawley rats were homogenized in ice cold buffer containing 25mM HEPES (pH 7.4), 0.25M sucrose, 5mM EDTA and 2mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4°C for 10 minutes at 10,000 X g followed by centrifugation at 50,000 X g for 45 minutes, and the resulting microsomal pellet was resuspended and stored at -70°C. For experiments, the microsomal membranes were solubilized in 2% Triton X-100 containing 2 mg/ml bacitracin and 2mM PMSF. After separation of non-soluble material by centrifugation, the supernatant was purified by wheat germ agarose chromatography as previously described (10,11). The average insulin binding capacity, measured according to (12) and estimated by Scatchard analysis (13), of the preparations was 18 pmol insulin bound per mg of protein. Because of previously reported technical difficulties in measuring EGF binding to solubilized receptors (14), no such measurements were conducted. Instead, binding of insulin and EGF to microsomal membranes was determined essentially as described by Carpenter et al. (15). The average binding capacity estimated by Scatchard analysis was 0.33 pmol EGF/mg and 2.5 pmol insulin/mg. Under the assumption that the recoveries of EGF and insulin receptors were similar, the specific EGF binding capacity in the wheat germ lectin purified preparation could be estimated from these data to be about 2.4 pmol/mg protein.

Phosphorylation Assays: Aliquots of the receptor preparations, containing 7 µg of protein, were preincubated in a total volume of 40 µl with no added hormone (control) or with saturating concentrations of insulin (170 nM) or EGF (100 nM) for 40 minutes at 23°C. The preparations were then chilled down to 4°C and substrate or water (10 µl) was added. Phosphorylation reactions were initiated by adding 10 µl of a mixture calculated to give final concentrations of 5mM $MnCl_2$, 12mM $MgCl_2$ and 50 uM [γ-^{32}P]ATP (1-5 µCi/nmol) and carried on at 4°C for various periods of time.

In experiments measuring the phosphorylation of the insulin receptor, EGF receptor, casein or histone, the reactions were terminated by addition of 10mM ATP, 100mM dithiothreitol, 10mM EDTA, 1% $NaDodSO_4$, 10% glycerol, 0.05% bromphenol

blue and 0.5M Tris base (final concentrations) and heating to 95°C for 3 minutes. Phosphorylated proteins were analyzed by SDS-PAGE (7.5% or 12.5% resolving) according to Laemmli (16). The gels were stained, dried, and the major phosphorylated bands, identified by autoradiography, were cut out and the radioactivity measured by liquid scintillation. The radioactivity in other pieces of the gel, judged by autoradiography to be free of discrete proteins, were subtracted as background.

In experiments measuring the phosphorylation of peptides or Glu4:Tyr1, phosphorylation reactions were terminated by addition of unlabeled ATP (final concentration 50mM). Aliquots of the reaction mixtures were spotted on squares of phosphocellulose paper (peptides) and washed in 75mM H_3PO_4 (17) or spotted on squares of Whatman 3 MM paper (Glu4:Tyr1) and washed in 10% trichloracetic acid (18). The radioactivity of the dried papers was measured by scintillation counting.

RESULTS

Phosphorylation of Endogenous Proteins: In wheat germ lectin purified preparations of solubilized rat liver membranes, insulin stimulated the phosphorylation of a 95KDa protein and EGF the phosphorylation of a 170KDa protein (Fig. 1). Previous studies on similar material have shown that the 95KDa- and 170KDa protein can be specifically immunoprecipitated by antiserum to the insulin receptor (1,11) and EGF receptor (2), respectively, confirming their identities as the insulin and EGF receptor. The time course for the phosphorylation of the 95KDa protein is shown in Fig. 2A. Insulin increased the initial rate of phosphorylation about 15-

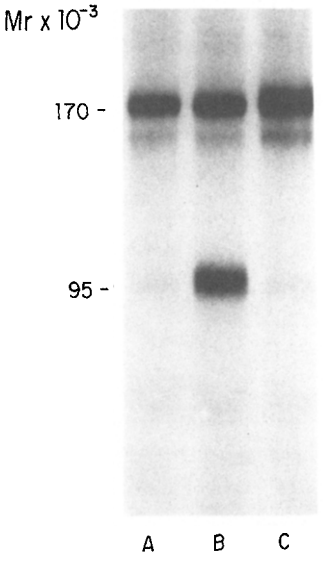

FIGURE 1: Autoradiogram showing the stimulation of protein phosphorylation by insulin and EGF. Portions of a wheat germ lectin purified receptor preparation were preincubated without hormone (A), 170nM insulin (B) or 100 nM EGF (C). Phosphorylation was carried out for 10 min as described under "Materials and Methods".

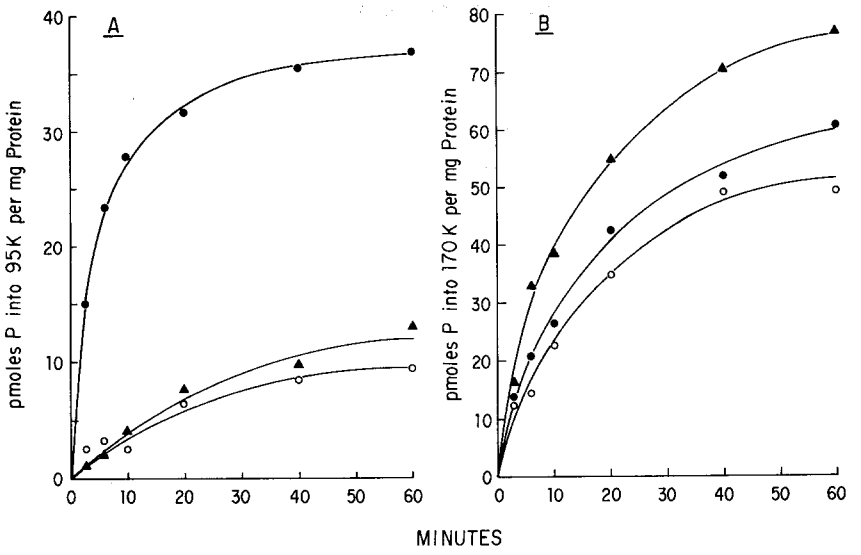

FIGURE 2: Time courses of insulin and EGF stimulated receptor phosphorylation. Portions of a partially purified receptor preparation were preincubated without hormone (o), 170nM insulin (●) or 100 nM EGF (▲). Phosphorylation reactions were carried out for various periods of time as described under "Materials and Methods". After SDS-PAGE and autoradiography, bands representing the 95KDa- and 170KDa-proteins were cut out and the radioactivity measured by liquid scintillation. Fig. 2A shows time courses for the insulin receptor, Fig. 2B for the EGF receptor.

fold and maximal levels of phosphorylation, in the presence or absence of insulin, were reached within 60 minutes. EGF also had a minor, but reproducible effect to increase phosphorylation of the 95KDa protein (Fig. 2A). This effect became most apparent at the later time points but was only about 10% of the effect observed with insulin. Fig. 2B shows the time course for the phosphorylation of the 170KDa protein. EGF increased the initial rate by 2-2.5-fold and maximal levels of autophosphorylation were not reached as quickly as with the 95KDa-protein. Insulin stimulated the phosphorylation of the 170KDa protein to a modest degree, reaching 30-35% of the degree of phosphorylation induced by EGF.

Phosphorylation of Exogenous Substrates: The abilities of insulin and EGF receptor kinase to phosphorylate angiotensin II, Val^5-angiotensin II, Glu4:Tyr1, histone 2B, src-related peptide and casein were assessed. For all substances, phosphorylation was linear with time for at least 40 minutes, regardless of the presence or absence of hormone (data not shown). The effect of insulin and EGF on phosphorylation, measured in the first 30 minutes after initiating the reaction,

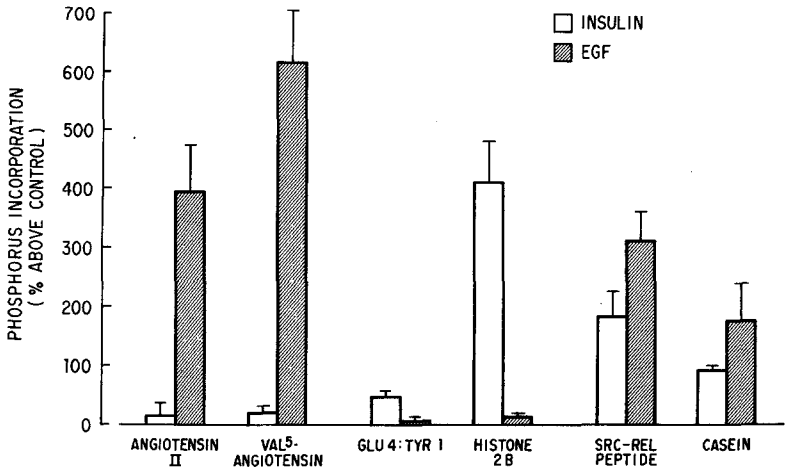

FIGURE 3: Effect of insulin and EGF on phosphorylation of exogenous substrates Portions of wheat germ lectin-purified preparations of solubilized rat liver membranes were preincubated with no added hormone, insulin (170nM) or EGF (100 nM). Phosphorylation reactions were carried out in the presence of histone (0.1 mg/ml) casein (0.3 mg/ml), angiotensin II (0.6mM), Val^5-angiotensin (0.4mM), src-related peptide (1.5mM) and Glu4:Tyr1 (0.2 mg/ml) as described under "Materials and Methods". Data are shown as percent of hormone stimulated phosphorylation above phosphorylation in the absence of hormone (control). Results represent mean values $\pm$ SEM of 3 experiments.

is shown in Fig. 3. The results indicate that the six substrates tested can be divided into three groups on the basis of their ability to serve as substrates for the insulin and EGF receptor kinases. Phosphorylation of angiotensin II and Val^5-angiotensin II was markedly increased by EGF, whereas insulin had only a small effect. On the other hand, phosphorylation of Glu4:Tyr1 and histone 2B were markedly stimulated by insulin, whereas EGF only had a minor effect. For src-related peptide and casein, both insulin and EGF were capable of stimulating phosphorylation. These results indicate that the insulin and EGF receptor kinase have different, but overlapping, substrate specificities.

When casein (Sigma No. 7891) was phosphorylated and was subjected to SDS-PAGE, two major protein bands (apparent Mr 32KDa and 29KDa) were detected. Interestingly, the insulin stimulated kinase favored the phosphorylation of the protein with apparent Mr 32KDa, whereas the EGF stimulated kinase favored phosphorylation of the protein with apparent Mr 29KDa (Fig. 4). This provides a further demonstration that the substrate specificities of insulin and EGF receptor kinases are different. When insulin and EGF were added together, the

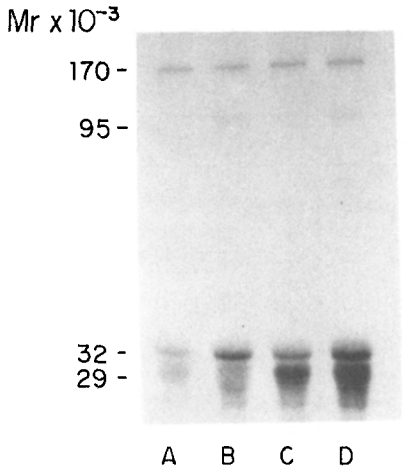

FIGURE 4: Phosphorylation of casein by insulin and EGF stimulated kinase. Portions of a partially purified receptor preparation were preincubated with no hormone added (A), 170 nM insulin (B), 100 nM EGF (C) or 160 nM insulin and 100 nM EGF (D). Phosphorylation reactions were carried out in the presence of a saturating concentration of casein (6 mg/ml) for 30 min as described under "Materials and Methods". Aliquots of the reaction mixtures were subjected to SDS-PAGE and autoradiography. The radioactivity (cpm) detected in the bands was:
apparent Mr = 29KDa: A:368, B: 1318, C: 1190, D: 1980
apparent Mr = 32KDa: A:692, B: 1492, C: 2871, D: 3619

effect of these hormones on casein phosphorylation was additive (Fig. 4, Lane D). We have found that the 29KDa band was not visualized on the gels when only the proteins precipitable in 3.3M urea at pH 4.6 were analyzed (data not shown). Consistent with previous studies (19,20), the 32KDa and 29KDa band represent α- and β-casein, respectively.

In Fig. 3, the effect of insulin and EGF on substrate phosphorylation is shown for a single non-saturating concentration of each substrate. Under these conditions, the extent of phosphorylation of a substrate could at least partly depend on the affinity of that substrate for the kinase, such that lack of phosphorylation could be due to low substrate affinity rather than an absolute specificity restriction. To assess this possibility, phosphorylation of each substrate was measured over a range of concentrations, and the Km and Vmax values for the phosphorylation of each substrate were determined (Table 1). These data demonstrate that, although some differences in the affinities of a given substrate for the two receptor kinases were apparent, the differences in substrate specificity of the two hormone receptor kinases existed over the

TABLE 1
KINETIC PARAMETERS FOR THE PHOSPHORYLATION OF EXOGENOUS SUBSTRATES BY EGF - AND INSULIN STIMULATED KINASES

	Control		Insulin		EgF	
	Km (μM)	Vmax (pmol/min/mg)	Km (μM)	Vmax (pmol/min/mg)	Km (μM)	Vmax (pmol/min/mg)
Angiotensin II	1710 ± 320	6.6 ± 0.7	2200 ± 490	10.3 ± 1.5	630 ± 0.1	22.4 ± 1.8
Val^5-Angiotensin	2250 ± 480	7.5 ± 0.6	3640 ± 620	10.5 ± 1.2	450 ± 60	20.2 ± 0.7
Glu4:Tyr1	5.0 ± 0.4	31.6 ± 0.6	6.3 ± 0.6	54.2 ± 1.4	4.6 ± 0.8	34.4 ± 1.6
Histone 2B	42.1 ± 5.8	2.4 ± 2.2	7.4 ± 2.2	5.2 ± 0.5	28.3 ± 5.1	2.5 ± 0.3
Src-related peptide	2110 ± 280	11.1 ± 0.8	2250 ± 460	40.6 ± 5.4	1450 ± 140	41.2 ± 2.0
Casein	10.5 ± 2.2	2.0 ± 0.1	10.3 ± 0.9	3.6 ± 0.1	11.3 ± 1.3	6.3 ± 0.3

Portions of a wheat germ lectin-purified preparation of solubilized rat liver membranes were pre incubated in the absence of hormone, 170 nM insulin or 100 nM EGF. Phosphorylation assays were carried out as described under "Materials and Methods" at 7 to 10 different concentrations of each substrate. Molar concentrations were calculated on the basis of Mr = 28,000 for Glu4:Tyr1, Mr = 13,770 for histone 2B and Mr = 23,000 for casein. Apparent Km and Vmax values ± SEM were calculated according to Wilkinson (26).

entire substrate concentration range, and in particular can be observed at maximal reaction rates (Vmax).

The data in Table 1 also indicate that for the substrates phosphorylated primarily by the EGF receptor kinase (angiotension II and Val^5-angiotensin II), EGF stimulates phosphorylation by decreasing the Km value and increasing the Vmax. For the substrates phosphorylated by both receptor kinases (src-related peptide and casein), both EGF and insulin predominantly increase the Vmax of the kinase reaction with little effect on the Km value. Finally, with substrates predominantly phosphorylated by the insulin receptor kinase (Glu4:Tyr1 and histone 2B), either an increase in Vmax (Glu4:Tyr1) or an increase in Vmax plus a decrease in Km (histone 2B) were observed.

DISCUSSION

As previously shown by other investigators (1,2,21), insulin and EGF stimulated the autophosphorylation of their respective receptors. To a lesser extent, EGF also increased the phosphorylation of the β-subunit of the insulin receptor and insulin stimulated the phosphorylation of the EGF receptor. This

phenomenon has also been observed in a partially purified receptor preparation from human placenta (9). Since previous reports have shown that at the hormone concentrations employed in the current study, essentially no insulin binds to the EGF receptor, and no EGF binds to the insulin receptor (22,23), a likely explanation for these findings is that insulin receptors can serve as a substrate for the EGF receptor kinase and vice versa. Recent studies suggest that phosphorylation of specific sites on the insulin (21,24) and EGF receptor (25) activates the receptor kinases. Thus, insulin could indirectly stimulate the EGF receptor kinase and EGF could indirectly stimulate the insulin receptor kinase by "cross phosphorylation". Whether similar phosphorylation "cross talk" between these receptors exists in intact cells is unknown.

When the ability of insulin and EGF stimulated protein kinases to phosphorylate a series of exogenous substrates was examined, three different patterns were observed: with angiotensin II and Val^5-angiotensin II, EGF markedly stimulated phosphorylation, while insulin was much less effective; Glu4:Tyr1 and histone were readily phosphorylated by the insulin receptor kinase, with very little stimulation due to EGF; finally, phosphorylation of src-related peptide and casein was stimulated by both hormones to a comparable extent. At present, it is not clear if the small effects of EGF to stimulate histone and Glu4:Tyr1 phosphorylation and insulin to stimulate angiotensin and Val^5-angiotensin phosphorylation are direct or indirectly mediated by the phosphorylation "cross talk" mechanism discussed above. Thus, insofar as receptor "cross talk" is involved, the data presented in Fig. 3 slightly underestimate the actual differences in substrate specificities of the receptor kinases.

In a previous report (9), Pike et al. found less pronounced differences in substrate specificities between insulin and EGF receptors using a partially purified preparation of human placental membranes. This might be explained by a different selection of substrates tested or the different tissue source.

Most studies of the solubilized insulin receptor kinase are conducted with partially purified material, which also contains EGF receptor kinase. Therefore, for studies in which quantitative measurements of EGF or insulin receptor

kinase activity are necessary without the confounding influence of the other receptor kinase, it is advantageous to use substrates that are selectively phosphorylated by only one of the receptor kinases. For these purposes, histone 2B and Glu4:Tyr1 appear suitable for the insulin receptor kinase and the angiotensins are suitable for the EGF receptor kinase.

These differences in substrate specificities found between solubilized insulin and EGF receptor kinases suggest that similar differences might exist in vivo. As protein phosphorylation may be an early step in the signal transmission process for the actions of insulin and EGF, this specificity might be an important mechanism for the cell to specifically react to stimulation by either hormone. On the other hand, analogous to casein and src-related peptide, substrates may exist in vivo which are phosphorylated by both kinases, leading to overlapping cellular responses to insulin and EGF.

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