In vitro tyrosine phosphorylation studies on RAS proteins and calmodulin suggest that polylysine-like basic peptides or domains may be involved in interactions between insulin receptor kinase and its substrate

(purified system/HRAS protein/KRAS protein)

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ABSTRACT We have investigated the in vitro tyrosine phosphorylation of the HRAS and KRAS proteins by human placental insulin receptor kinase. Purified HRAS proteins are not phosphorylated by purified insulin receptor kinase. Since the tyrosine phosphorylation of calmodulin by the insulin receptor kinase in vitro requires cofactors such as protamine and poly(L-lysine), we examined the possibility that poly(L-lysine) may also potentiate the interaction between RAS proteins and the insulin receptor. We found that purified HRAS proteins are indeed phosphorylated by purified insulin receptor kinase in the presence of poly(L-lysine). In contrast, the KRAS protein, which carries an extremely basic domain (residues 172–182, Lys-Asp-Glu-Lys-Ser-Arg), is phosphorylated by the receptor kinase without the addition of basic proteins. We then determined whether the KRAS basic domain peptide plays a role similar to that of poly(L-lysine) and found that both the HRAS protein and calmodulin are phosphorylated by the receptor kinase in the presence of the KRAS basic domain peptide. Further examination of the role of poly(L-lysine) in potentiating tyrosine phosphorylation of the HRAS protein and calmodulin by purified insulin receptor kinase indicates that poly(L-lysine) affects the conformation of these protein substrates as well as that of the receptor kinase domain. These studies suggest that polylysine-like basic proteins or domains are required to establish the interaction between insulin receptor kinase and its substrate.

The RAS genes, HRAS, NRAS, and KRAS, have been studied intensively since their transforming alleles were first identified in human tumors in 1982. They code for structurally related proteins of 21,000 daltons (p21) that bind guanine nucleotides, have GTPase activity, and are associated with the plasma membrane (1). RAS proteins show significant sequence homology with guanine nucleotide binding (G) proteins (2). This evidence suggests that RAS proteins may participate in the transduction of signals across cellular membranes.

In an attempt to define the role of RAS proteins in transmembrane signaling systems, we have previously investigated a potential interaction between HRAS proteins and the insulin receptor and reported that insulin stimulates phosphorylation of the Harvey murine sarcoma virus transforming gene-encoded ras protein (v-Ha-ras) in isolated membranes (3). Consistent with our observation, others have suggested that microinjection of monoclonal antibodies against the ras protein inhibits insulin action in frog oocytes (4, 5). However, we were not able to show a direct interaction between purified Ha-ras and insulin receptor proteins (3). O’Brien et al. (6) have also observed that the insulin receptor does not phosphorylate native Ha-ras protein.

The phosphorylation of calmodulin by the insulin receptor in vitro has been found to require cofactors such as protamine and poly(L-lysine) (7) while in intact adipocytes calmodulin appears to be phosphorylated at tyrosine residues in an insulin-dependent manner (8). More recently, we have studied the role of poly(L-lysine) in phosphorylation of calmodulin and src-related peptide by purified insulin receptor kinase (9, 10). [A synthetic peptide resembling the tyrosine phosphorylation site of pp60Src (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) is routinely used to measure tyrosine-specific protein kinase activity (11).] We report here that poly(L-lysine) potentiates the tyrosine phosphorylation of purified HRAS proteins by the insulin receptor kinase in vitro. Based on these findings we predicted that, in contrast to the HRAS protein, the KRAS protein, which contains a poly(L-lysine)-like domain in its carboxyl terminal region, would interact directly with the insulin receptor. We found that the KRAS protein is indeed phosphorylated by the receptor kinase in vitro. We also found that a basic peptide from the KRAS protein potentiates the interaction between the receptor kinase and its substrate. We have further examined modalities of interaction between the receptor kinase, poly(L-lysine), and either substrate (HRAS or calmodulin), and we propose a model explaining our findings.

MATERIALS AND METHODS

Materials. Insulin receptor was purified 2400-fold from human placental membranes by sequential affinity chromatography on wheat germ agglutinin- and insulin-Sepharose as described (12). The receptor was apparently pure and retained both insulin binding activity and tyrosine kinase activity (12–14). Human normal (wt) and oncogenic (T24) HRAS proteins were expressed in an Escherichia coli expression system (provided by R. Sweet, Smith Kline & French) (15) and were purified to homogeneity as described (3). Human v-KRAS protein expressed in E. coli was purified to

Abbreviations: wt, wild-type HRAS protein; T24, T24 transforming (Gly → Val at position 12) HRAS protein; BSA, bovine serum albumin.

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homogeneity as described (16). A peptide corresponding to the last 14 amino acids in the carboxyl terminus of the human c-KRAS2 gene product (Lys6-Ser-Lys-Thr-Lys-Cys-Val-Leu-Met) was provided by K. C. Glenn (Monsanto). Poly(t-lysine) (Mw 30,000–70,000), γ-globulin (IgG), β-galactosidase, myosin, phosphorylase b, bovine serum albumin (BSA), and phosphoamino acids were obtained from Sigma. Purified porcine brain calmodulin was from Ocean Biologics (Edmonds, WA). Crystalline porcine insulin was supplied by Eli Lilly. Molecular weight markers and reagents for NaDodSO4/PAGE were purchased from Bio-Rad. [γ-32P]ATP was from New England Nuclear.

**Phosphorylation of Protein Substrates by Purified Insulin Receptor Kinase.** The standard condition was that the purified insulin receptor kinase (0.05–0.1 μg) and the indicated amounts of protein substrates, such as purified RAS protein or calmodulin, were incubated for 60 min at 25°C in the absence or presence of 0.1 μM insulin and/or 0.24 μM poly(t-lysine) in 12.5 μl of 50 mM Tris-HCl buffer, pH 7.4/0.1% Triton X-100. The phosphorylation reaction was initiated by the addition of 2.5 μl of 6 times-concentrated [γ-32P]ATP/metal ion mixtures that gave final concentrations of 40 μM ATP (=12,000 cpm/pmol), 2 mM MnCl2, and 15 mM MgCl2. The reaction was allowed to proceed for 20 min at 25°C and stopped by addition of 7.5 μl of 3 times-concentrated Laemmli sample buffer, followed by boiling for 5 min. NaDodSO4/PAGE was performed according to Laemmli (17) under reducing conditions.

**RESULTS**

**Phosphorylation of HRAS Proteins by Purified Insulin Receptor Kinase in the Absence or Presence of Poly(t-lysine).** To examine the possibility that poly(t-lysine) may potentiate the interaction between HRAS proteins and the insulin receptor kinase, we used both normal (glycine at position 12; wt) and oncogenic (valine at position 12; T24) RAS proteins purified from *E. coli* cells that stably expressed one or the other RAS protein. As shown in Fig. 1 (lanes 3 and 5), the purified insulin receptor kinase did not phosphorylate either RAS protein. Phosphorylation of the RAS proteins was not observed even in the presence of insulin (data not shown). [We have previously shown that the receptor kinase does not phosphorylate calmodulin in the absence or presence of insulin (9).] However, the HRAS proteins were phosphorylated by the purified insulin receptor kinase in the presence of poly(t-lysine) (lanes 4 and 6). Incubation of RAS proteins with poly(t-lysine) and BSA (in place of the receptor kinase) did not cause phosphorylation of the RAS proteins, indicating that the poly(t-lysine)-induced phosphorylation of RAS proteins is not due to some other protein kinase contaminating the RAS protein preparations (lanes 7 and 8). As we have reported (10), poly(t-lysine) stimulates phosphorylation of the β subunit as well as the α and β1 subunits of the insulin receptor (Fig. 1, lane 2). The β1 subunit, which is often found in purified insulin receptor preparations, is the N-terminal portion of the β subunit generated by proteolytic loss of the kinase domain (18, 19). Of interest also is that phosphorylation of the receptor β subunit was seemingly enhanced in the presence of the RAS protein (lanes 3 and 5).

Phosphoamino acid analysis has shown that both the HRAS proteins and the insulin receptor β subunit are phosphorylated exclusively on tyrosine residues (data not shown).

**Effect of Poly(t-lysine) on Phosphorylation of Various Proteins by Purified Insulin Receptor Kinase.** To test the possibility that poly(t-lysine) may be able to act on acidic proteins other than the RAS proteins and calmodulin, various proteins that are not known to be substrates for the kinase were examined. IgG, β-galactosidase, myosin, and phosphorylase b (data not shown) as well as BSA (Fig. 2, lanes 8 and 10). The experiments showed that myosin, phosphorylase b, and BSA are not good substrates even in the presence of poly(t-lysine). β-Galactosidase appeared to be phosphorylated by the insulin receptor kinase in the absence of poly(t-lysine) and this phosphorylation was enhanced when the receptor was activated in the presence of poly(t-lysine). The IgG heavy chain seemed to be slightly phosphorylated by the receptor kinase in the presence of poly(t-lysine), but the intensity of phosphorylation was much lower than that of receptor autophosphorylation or phosphorylation of the RAS proteins or calmodulin.

**Phosphorylation of the Purified KRAS Protein by Purified Insulin Receptor Kinase.** Since the KRAS protein has a poly(t-lysine)-like domain in its carboxyl-terminal variable domain (residues 172–182, Lys-Asp-Glu-Lys6-Ser-Arg), we
hypothesized that the KRAS protein may be phosphorylated by the insulin receptor kinase in the absence of cofactor basic proteins. Purified KRAS protein was phosphorylated by purified insulin receptor kinase (Fig. 3, lane 1) and this phosphorylation was enhanced in the presence of insulin (lane 2) whereas in control experiments phosphorylation of the HRAS protein by the receptor kinase was observed only in the presence of poly(L-lysine) (lanes 3 and 4).

**Phosphorylation of the HRAS Protein and Calmodulin by Purified Insulin Receptor Kinase in the Presence of the KRAS Basic Domain Peptide.** To examine the ability of the poly(L-lysine)-like basic domain found in the KRAS protein to link insulin receptor kinase with its substrates, a peptide corresponding to the carboxyl-terminal domain of the c-KRAS2 protein (Lys6-Ser-Lys-Thr-Lys-Cys-Val-Leu-Met) (20) was used in place of poly(L-lysine). As shown in Fig. 4, both substrates were phosphorylated in the presence of the KRAS peptide. Higher concentrations of the peptide were required for the phosphorylation as compared to poly(L-lysine): 20–200 μM vs. 0.1–1 μM. In addition, the peptide was less effective than poly(L-lysine); phosphorylation in the presence of the KRAS peptide, although somewhat variable, averaged 2% and 9% of the effects achieved by poly(L-lysine) for calmodulin and HRAS protein, respectively.

**Fig. 3.** Phosphorylation of the purified KRAS protein by purified insulin receptor kinase. KRAS protein (=2 μg) was incubated with insulin receptor kinase (IR; ≈0.1 μg) in the absence (lane 1) or presence (lane 2) of insulin (INS). Lanes 3 and 4: controls in which the HRAS protein (wt, ≈2 μg) was incubated with insulin receptor kinase (≈0.1 μg) in the presence of insulin (lane 3) or 0.24 μM poly(L-lysine) (PLL) (lane 4). The phosphorylation reaction was performed as described in Materials and Methods. Samples were analyzed by NaDodSO4/10% PAGE and autoradiography.

**Fig. 4.** Phosphorylation of the HRAS protein (A) and calmodulin (B) by purified insulin receptor kinase in the presence of the KRAS basic peptide. Insulin receptor kinase (≈0.1 μg) and HRAS protein (1 μg) or calmodulin (4 μg) were incubated in the presence of poly(L-lysine) (PLL) or the KRAS basic peptide and 0.1 μM insulin at 25°C for 60 min. The phosphorylation reaction was performed as described in Materials and Methods except that the calmodulin reaction mixtures contained 1 mM EGTA since the presence of Ca2+ inhibits the phosphorylation of calmodulin (9). Samples were analyzed by NaDodSO4/12.5% PAGE. The gels were exposed to Kodak XAR 5 film with one intensifying screen for 1 hr (B, lane 1), 5 hr (A, lane 1; B, lanes 2–7), or 15 hr (A, lanes 2–7).

**Phosphorylation of the HRAS Protein and Calmodulin by Purified Insulin Receptor Kinase After Various Preincubation Conditions.** In our standard experiments, a substrate, the insulin receptor, insulin, and poly(L-lysine) were preincubated, and the phosphorylation reaction was initiated by addition of [γ-32P]ATP/metal ion mixtures. When the preincubation conditions were varied, 32P incorporation into the RAS protein or calmodulin was strikingly different from that obtained under the standard preincubation condition. A representative experiment is shown in Fig. 2, and a summary of these experiments is presented in Table 1. A scheme derived from these results is illustrated in Fig. 5.

Lanes 1–4 and 11–14 of Fig. 2 show the phosphorylation of calmodulin and the HRAS protein, respectively, under different conditions. Of the four preincubation conditions used, that shown in lanes 1 and 11 appears to be best for phosphorylation of either substrate by the receptor kinase. Under this condition, substrate, receptor, and insulin were preincubated first, then poly(L-lysine) was added just before the phosphorylation reaction was initiated. Hence, we used 32P incorporation into either substrate under this condition as a

### Table 1. Comparison of phosphorylation of the HRAS protein, calmodulin, and the insulin receptor β subunit by purified insulin receptor kinase under various preincubation conditions

<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Addition just prior to reaction</th>
<th>Phosphorylation of substrate, %</th>
<th>Phosphorylation of β subunit in presence of substrate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fig. 2 lanes</td>
<td>Calmodulin</td>
<td>HRAS</td>
</tr>
<tr>
<td>1. Substrate + IR + Ins*</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Substrate + IR + PLL</td>
<td>1 and 11</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3. Substrate + IR + PLL</td>
<td>None</td>
<td>48.8 ± 19.4</td>
<td>89.6 ± 16.1</td>
</tr>
<tr>
<td>4. IR + Ins + PLL</td>
<td>Substrate</td>
<td>24.2 ± 7.7</td>
<td>14.4 ± 7.7</td>
</tr>
<tr>
<td>5. Substrate + PLL, IR + Ins</td>
<td>4 and 14</td>
<td>32.7 ± 7.1</td>
<td>55.6 ± 3.3</td>
</tr>
</tbody>
</table>

Experimental conditions are described in the legend to Fig. 2. Substrates were wt HRAS or calmodulin. Ins, insulin; IR, insulin receptor; PLL, poly(L-lysine). Phosphorylation was determined by excision of the appropriate band from the gel followed by quantitation of incorporated radioactivity by Cerenkov counting. Numbers in parentheses represent numbers of experiments.

*Phosphorylation results extrapolated from many identical experiments as shown in Fig. 1 (lanes 3 and 5) and Fig. 2 (lane 9).
Fig. 5. Speculative schematic of our present and previous studies on the insulin receptor kinase. The insulin kinase β subunit is shown in the upper center (form I) with the kinase domain illustrated as a “horseshoe” shape with its catalytic site on the bottom, and four alternative conformations of the catalytic site, implying different degrees of kinase activity, are shown on the lower right. The boxes indicate the transmembrane domain (TM) and the acidic A-III domain residues (1268-1286), which is the potential site for the poly(L-lysine) interaction previously proposed (10). The carboxyl-terminal region downstream from the A-III domain is comprised of 57 amino acid residues. This region contains a short stretch of basic domain (residues 1329-1333; Lys-Asn-Gly-Arg), which is indicated as a “knot.” Insulin receptor kinase inactivation occurs as illustrated by the sequence I → IV → V (21). Poly(L-lysine) activates both intact (form I) and partially inactivated (form IV) kinases to give form II (10). Neither the H-RAS protein nor calmodulin can be phosphorylated by the purified insulin receptor kinase alone; I → VI (3, 9). When poly(L-lysine) is added to the H-RAS (or calmodulin)/receptor kinase mixture, the H-RAS protein (or calmodulin) is phosphorylated by the receptor kinase; VI → VII (this study and ref. 9). When substrates are added to the receptor kinase–poly(L-lysine) complex, phosphorylation of the H-RAS protein or calmodulin is greatly reduced; II → III. When the substrates and poly(L-lysine) are preincubated first and the receptor kinase is added later, the degree of phosphorylation of the substrates is decreased as compared to the controls; I → VIII (this study).

control (Table 1, condition 2; 100%) (form VII in Fig. 5). In the case of HRAS protein phosphorylation, the standard condition (lane 12) gave results approximately equal to those of the control condition (Table 1, condition 3; 89.6 ± 16.1%). In contrast, calmodulin phosphorylation was significantly reduced under this condition (Fig. 2, lane 2, and Table 1, condition 3; 48.8 ± 19.4%). When the receptor, insulin, and poly(L-lysine) were preincubated first, and the substrates added just before initiation of the phosphorylation reaction, HRAS protein phosphorylation was significantly reduced (Fig. 2, lane 13, and Table 1, condition 4; 14.4 ± 7.7%) and calmodulin phosphorylation was also lower than that obtained under the standard condition (Fig. 2, lane 3, and Table 1, condition 4; 34.2 ± 7.7%) (form III in Fig. 5). When the substrates and poly(L-lysine) were preincubated together and the receptor and insulin were preincubated together, and these mixtures were combined just before initiation of the phosphorylation reaction, the degree of phosphorylation of calmodulin and the HRAS protein was significantly lower than controls (Fig. 2, lane 4, and Table 1, condition 5; 32.7 ± 7.1%; Fig. 2, lane 14, and Table 1, condition 5; 55.6 ± 3.3%, respectively) (form VIII in Fig. 5).

Fig. 2 also shows the effects of poly(L-lysine) and substrates on the insulin receptor itself. Phosphorylation of the β subunit appeared to be enhanced in the presence of calmodulin or the RAS protein (Figs. 1 and 2). The degree of β-subunit phosphorylation under different preincubation conditions (Fig. 2) is summarized in Table 1. These results indicate that interaction between the insulin receptor kinase and these substrates increase the kinase activity.

**DISCUSSION**

We have shown that purified HRAS proteins are phosphorylated by purified insulin receptor kinase in the presence of poly(L-lysine), whereas the purified KRAS protein is phosphorylated by the receptor kinase without the addition of basic proteins. The difference observed between the two RAS proteins can most likely be attributed to the amino acid sequence differences in their carboxyterminal domains (I). The human KRAS protein carries an extremely basic domain at residues 172-182 (Lys-Asp-Glu-Lys(Ser)-Arg), whereas this domain in the human HRAS and N-RAS protein consists of primarily neutral amino acid residues. Our studies using purified insulin receptor and RAS proteins suggested the possibility that RAS proteins may be phosphorylated at tyrosine residues in intact cells, although to our knowledge, this has not been reported.

In this study, we have examined the molecular interactions between insulin receptor kinase and potential substrates in vitro. There always seems to be a critical gap between studies using intact cells and those using broken cell preparations. In other words, even if two protein components are shown to interact in an intact cell system, it is unlikely that an identical in vitro system can be established simply by mixing the purified protein components. We know that at least two insulin-sensitive phosphorylation systems, the HRAS pro-
teins and calmodulin, have this characteristic. In both cases, phosphorylation of the proteins has been observed in an insulin-dependent manner in intact cells, but when the purified proteins or calmodulin were incubated with purified insulin receptor kinase, neither were phosphorylated (3, 9). Whether or not calmodulin is directly phosphorylated by insulin receptor kinase in intact cells requires further investigation, since although Colca et al. (8) have reported that calmodulin is phosphorylated in an insulin-dependent manner in intact rat adipocytes, Blaeskhe and Haupt (22) have recently provided evidence that calmodulin is not phosphorylated in 3T3-L1 adipocytes. This in vitro study, as well as previous studies (7, 9) suggests that a poly(t-lysine)-like basic protein or domain is required for establishment of the direct interaction between the insulin-responsive protein and the insulin receptor in broken cells or purified systems.

Polylysine-containing peptides have been shown to affect membrane-associated enzymes (20). Gatica et al. (20) have searched for protein sequences containing long stretches of lysine residues and found that the human Kras2 gene has six consecutive lysine residues. A peptide corresponding to the last 14 amino acids in the carboxyl terminus of the human Kras2 gene product was synthesized, and it also stimulated in vitro membrane protein phosphorylation. We hypothesized that this peptide might be a naturally occurring polylysine-like peptide, acting as a cofactor for phosphorylation of the HRAS protein and calmodulin. We found that the Kras peptide, like poly(t-lysine), is effective in mediating phosphorylation of both of these substrates by insulin receptor kinase. These studies suggest that the basic domains of naturally occurring proteins may play a role in establishing interactions between the receptor kinase and its substrates.

We have proposed a model for the interactions among poly(t-lysine), insulin receptor kinase, and either substrate (Fig. 5). This model represents one of the several possibilities which will require further experiments to verify. Although we cannot exclude a possible allosteric regulation on kinase activity by poly(t-lysine), the results of the various preincubation-condition experiments (Table I) would most likely fit the hypothesis described here. Our previous studies suggest that the conformation of the carboxyl-terminal domain of the receptor kinase becomes relaxed (form IV) during purification and storage and that, after this conformational change occurs, the carboxyl-terminal α20-α20 amino acid domain is readily proteolysed, resulting in form V (21, 23). The kinase activity of the relaxed form (IV) is ~10% of that of the native form (I), and this form (IV) can no longer be activated by insulin (21). Our recent studies (10) have suggested the following. (i) The native kinase (form I) and the relaxed kinase (form IV) may both be activated by incubation with poly(t-lysine). We have proposed that poly(t-lysine) activates the kinase by interaction with three acidic domains of the insulin receptor, including residues 1268–1268 (an acidic A-III domain) (10), to give an activated kinase (form II) that is much more active than the native form (I; 10- to 50-fold). (ii) The form (V) that has lost the carboxyl-terminal α20 amino acids is an inactive kinase and the A-III domain may be such a conformation that poly(t-lysine) is no longer able to restore the kinase activity.

We have also shown in the present and in a previous study (9) that the purified insulin receptor kinase (form I) is not able to phosphorylate calmodulin or the RAS protein as illustrated in form VI. However, after addition of poly(t-lysine), the RAS protein (or calmodulin) can become phosphorylated by the kinase as illustrated in form VII. Although the molecular mechanism underlying this result is not yet clear, one possibility is that poly(t-lysine) binds to the A-III domain and consequently changes the conformation of the substrates (form VII). Our previous study also showed that poly(t-lysine) binds directly to both the insulin receptor and calmodulin and that calmodulin changes its conformation by interaction with poly(t-lysine) as determined by circular dichroism spectra (9).

The poly(t-lysine)-activated kinase (form II) alone does not phosphorylate either the HRAS protein or calmodulin very effectively (form III). This indicates that poly(t-lysine) may bind very closely to the kinase domain and that the protein substrate is not able to fit into the catalytic domain, whereas the src-related peptide can easily fit and therefore be phosphorylated (10). When substrates and poly(t-lysine) are incubated first and then mixed with the kinase (form VIII), the degree of phosphorylation of the HRAS protein is between those observed under the other two conditions (forms VII and II) whereas calmodulin phosphorylation is at nearly the same level as that shown in form III. This possibly reflects the structural difference between the two substrates. These results suggest that the substrate–poly(t-lysine) complex cannot fit as well into the kinase domain (form VIII) as compared to form VII.

These studies have focused on a potential role of basic protein cofactors in mediating the interaction between the insulin receptor kinase and insulin-responsive protein substrates. The studies provide an in vitro system useful for identification of naturally occurring cofactors, as we have shown for the basic HRAS peptide. Furthermore, functional changes in the activities of tyrosine-phosphorylated RAS proteins might best be investigated using an experimental system of this type.

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