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# Aggregation of IGF-I Receptors or Insulin Receptors and Activation of Their Kinase Activity Are Simultaneously Caused by the Presence of Polycations or K-ras Basic Peptides†

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ABSTRACT: Several groups including us reported that basic proteins and polycations activate the insulin receptor tyrosine-specific protein kinase (TPK) in vitro. However, some inconsistency has become obvious in the observations. The most intriguing was the brief description by Morrison et al. [(1989) *J. Biol. Chem. 264*, 9994–10001] that polylysine had no effect on the IGF-I receptor TPK despite its 84% identity to the insulin receptor TPK. In the present study, we used highly purified IGF-I and insulin receptor TPKs in an effort to solve the discrepancies noted in the recent publications and to reveal the mechanism by which polycations stimulate the receptor TPKs. We report that the IGF-I receptor TPK is stimulated by polycations and basic proteins in a manner similar to their effects on the insulin receptor TPK. When effects of polylysine and polyarginine on both receptor TPKs were closely compared, subtle qualitative differences were found: Polylysine stimulated autophosphorylation and exogenous substrate phosphorylation activities of both insulin receptor TPK and IGF-I receptor TPK similarly. In contrast, another polycation, polyarginine, affected both TPKs in a manner quite different from polylysine: Polyarginine stimulated insulin receptor autophosphorylation to a greater extent than polylysine did while it had a very small effect on the IGF-I receptor autophosphorylation as well as the exogenous substrate phosphorylation activities of the two receptor TPKs. We have further extended the studies to include the domains of natural proteins which contain a polylysine-like sequence. Such peptides, K-ras peptides, had similar effects on the two receptor TPKs to those of polycations. Finally, we observed that polycations and K-ras peptides caused receptor aggregation as judged by nondenaturing gradient polyacrylamide gel electrophoresis and sucrose density gradient centrifugation. These studies suggest a strong correlation between receptor aggregation and activation of IGF-I and insulin receptor TPKs which may be caused by cellular proteins such as K-ras products.

Insulin-like growth factor I (IGF-I)[1] and insulin receptors are membrane-bound glycoproteins that are composed of two extracellular $\alpha$ subunits and two transmembrane $\beta$ subunits. The $\beta$ subunit carries cytoplasmic tyrosine-specific protein kinase (TPK) activity. Ligand binding to the receptor extracellular domain induces autophosphorylation of the $\beta$ subunit and activation of the TPK. This TPK activation process is required for some or all of IGF-I's and insulin's transmembrane signaling (Rosen, 1987; Goldfine, 1987; Roth et al., 1988; Czech, 1989). Hence, further characterization of the TPKs is essential to understand the signal transduction mechanisms of IGF-I and insulin.

We and others recently reported that basic proteins and polycations activate the insulin receptor TPK in vitro (Fujita-Yamaguchi et al., 1989a; Sacks et al., 1989a; Sacks & McDonald, 1988; Rosen & Lebwohl, 1988; Morrison et al., 1989; Kohanski, 1989). Sacks et al. first observed that protamine and polylysine activate phosphorylation of the $\beta$ subunit (Sacks et al., 1989a; Sacks & McDonald, 1988). Rosen and Lebwohl postulated that polylysine alters divalent cation requirements of the insulin receptor TPK (Rosen & Lebwohl, 1988) whereas we suggested that polylysine directly interacts with the $\beta$ subunit, thereby activating the receptor TPK independently of insulin (Fujita-Yamaguchi et al., 1989a). Morrison et al. characterized effects of polylysine and divalent metals on insulin and IGF-I receptor TPKs (Morrison et al., 1989). In contrast to our results, they observed that polylysine activates insulin receptor TPK only in the presence of insulin. In addition, they found that polylysine was completely unable to stimulate IGF-I receptor TPK. In light of the structural homology between the two TPKs, 84% identity at the amino acid sequence level (Ullrich et al., 1986), this striking difference in the polylysine effect on the two TPKs reported by Morrison et al. (1989) required further verification. Finally, Kohanski reported that both polylysine and polyarginine

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[1] Abbreviations: TPK, tyrosine-specific protein kinase; IGF-I, insulin-like growth factor I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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stimulate autophosphorylation of the insulin receptor in the absence or presence of insulin (Kohanski, 1989), which appeared somewhat contradictory to our previous observation that unlike polylysine, polyarginine inhibits phosphorylation of an exogenous substrate by the insulin receptor TPK (Fujita-Yamaguchi et al., 1989a). The potential discrepancy lying between his and our observations needed to be investigated further.

Aggregation of cell surface receptors has been documented. Earlier studies include cytochemical observations such as microaggregation and patching of the receptor molecules induced by ligands and receptors (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978; Schechter et al., 1979). Anti-receptor antibodies are able to mimic biological effects of the hormone (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978; Schechter et al., 1979; Schreiber et al., 1981). Monovalent (Fab') antibody lacks the ability to initiate the biological responses while cross-linking of anti-receptor Fab's with anti-IgG restores this ability (Kahn et al., 1978; Schreiber et al., 1983). These studies suggested that microaggregation of receptors is required for signal transduction. Similar aggregation phenomena have been described for broken cell systems as well. The oligomerization state of the insulin receptor and its activity have been analyzed by nondenaturing polyacrylamide electrophoresis (Kubar & Obberghen, 1989), sucrose density gradient centrifugation (Kohanski, 1989), and radiation inactivation techniques (Fujita-Yamaguchi et al., 1989). These studies have indicated that an aggregated form of the insulin receptor exhibits high autophosphorylation (Kohanski, 1989; Kubar & Obberghen, 1989; Fujita-Yamaguchi et al., 1989) or kinase activities (Fujita-Yamaguchi et al., 1989).

In the present study, we used highly purified IGF-I and insulin receptor TPKs in order to answer the questions raised from the aforementioned publications and to reveal the mechanism by which polycations stimulate the receptor TPKs. We report that the IGF-I receptor TPK is stimulated by polycations and basic proteins in a manner similar to their effects on the insulin receptor TPK. When effects of polylysine and polyarginine on both receptor TPKs were closely compared, subtle qualitative differences were found. We have extended the studies to include the domains of natural proteins which contain a polylysine-like sequence. Such peptides, K-ras peptides, had similar effects on the two receptor TPKs to those of polycations. As a potential mechanism by which polycations and K-ras peptides activate the receptor TPKs, we show the evidence that polycations and K-ras peptides promote receptor aggregation as judged by nondenaturing polyacrylamide gel electrophoresis and sucrose density gradient centrifugation. These studies suggest a strong correlation between aggregation and activation of IGF-I or insulin receptor TPK which may be caused by cellular proteins such as K-ras products.

## Experimental Procedures

*Materials.* IGF-I receptor and insulin receptor were purified to apparent homogeneity from human placentas as described (Fujita-Yamaguchi et al., 1983; LeBon et al., 1986; Fujita-Yamaguchi & LeBon, 1990). Poly(L-lysine) (average $M_r$ 3800, 12000, 41000, and 289000),[2] poly(L-arginine) (average $M_r$ = 41000), and protamine sulfate were purchased from Sigma. Crystalline porcine insulin was a gift from Eli Lilly. Peptides corresponding to the last 14 amino acids in the carboxyl terminus of human K-ras 2B ($Lys_6$-Ser-Lys-Thr-Lys-Cys-Val-Ile-Met) and rat K-ras ($Lys_6$-Ser-Arg-Thr-Arg-Cys-Ile-Val-Met) (Shimizu et al., 1983) were synthesized by Dr. B. Kaplan of our department. A synthetic peptide resembling the tyrosine phosphorylation site of $pp60^{src}$ (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, RR-src peptide) was purchased from Peninsula Laboratories. Control peptides (Cys-Ser-Ser-Thr-Ser-Val-Asp-Thr-Ser-Pro-Glu-Glu-His-Arg and Cys-Glu-Ser-Glu-Asp-Ser-Gly-Lys-His-Asn-Gly-Ser-Glu-Tyr-Glu) were gifts from Dr. J. Ramachandran (Genentech). [$\gamma$-$^{32}$P]ATP was from New England Nuclear. Molecular markers were obtained from Pharmacia and Bio-Rad.

*Phosphorylation of an Exogenous Substrate (RR-src Peptide Phosphorylation).* Purified receptors (0.01–0.1 $\mu$g) and indicated amounts of kinase effectors, such as polycations, IGF-I, insulin, or K-ras peptides, were incubated for 40 min at 25 °C in 10 $\mu$L of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100. To this solution was added 2.5 $\mu$L of 6 mM RR-src peptide, and the phosphorylation reaction was initiated by the addition of 2.5 $\mu$L of 6 times concentrated [$\gamma$-$^{32}$P]ATP–metal ion mixtures that gave final concentrations of 40 $\mu$M ATP (12000 cpm/pmol), 2 mM $MnCl_2$, and 15 mM $MgCl_2$. The reaction was allowed to proceed for 20 or 40 min at 25 °C and stopped by the addition of 25 $\mu$L of 5% trichloroacetic acid and 10 $\mu$L of 1% bovine serum albumin. After incubation of this solution at 0 °C for 15 min, the proteins were precipitated by centrifugation. Duplicate 17-$\mu$L aliquots of the supernatant were spotted onto pieces of phosphocellulose paper (Whatman, P-81). The papers were extensively washed in 75 mM phosphoric acid. Incorporation of $^{32}$P into the peptide was quantified by liquid scintillation counting.

*Phosphorylation of the $\beta$ Subunit and SDS–PAGE.* Phosphorylation of the purified receptor was carried out basically in the same manner as described for the exogenous substrate phosphorylation. The reaction was stopped by the addition of 7.5 $\mu$L of 3 times concentrated Laemmli sample buffer (Laemmli, 1970), followed by boiling for 5 min. SDS–PAGE was performed according to Laemmli under reducing or nonreducing conditions. The gels were stained with silver, dried, and autoradiographed.

*Nondenaturing Gradient Polyacrylamide Gel Electrophoresis (ND-PAGE).* ND-PAGE was carried out essentially as described by Kubar and Van Obberghen (1989). Briefly, 24 g of acrylamide and 1 g of bis(acrylamide) were dissolved in 100 mL of the electrophoresis buffer containing 90 mM tris(hydroxymethyl)aminomethane, 80 mM boric acid, 2.5 mM EDTA, and 0.3% Triton X-100, pH 8.4. A linear gradient of 0.5–15% acrylamide without a stacking gel was made using a mini-slab gel apparatus (Bio-Rad, 8 × 10 cm in size, 0.75-mm thickness). Sample buffer was prepared with 2 times concentrated electrophoresis buffer containing 27% sucrose and 0.01% bromphenol blue. After the phosphorylation reaction as described above, 12.5 $\mu$L of the sample buffer was added to the reaction mixture. The samples were immediately applied onto the gel which had been preelectrophoresed for 30 min at 100 V at 4 °C. Electrophoresis was usually carried out at 70 V for 15 h at constant voltage. The gel was first autoradiographed to examine whether or not radioactive materials had remained at the top of the gel, and then stained with silver or Coomassie Blue, dried, and autoradiographed again.

*Sucrose Density Gradient Centrifugation.* Gradients were prepared according to the methods described by Kohanski (1989) in a total volume of 4.7 mL. The gradients were

[2] An average $M_r$ = 41000 was usually used unless otherwise stated.

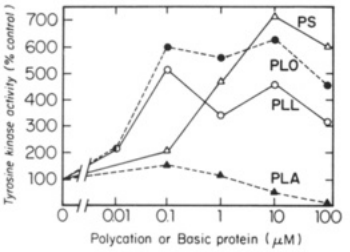


FIGURE 1: Effects of polycations and basic protein on IGF-I receptor kinase activity. Purified IGF-I receptor (~0.1 μg) was preincubated at 25 °C for 40 min with different concentrations of either polycation [PLO, poly(L-ornithine) (●); PLL, average $M_r$ = 41 000 poly(L-lysine) (○); and PLA, poly(L-arginine) (▲)] or protamine sulfate [PS (△)] as indicated. Tyrosine kinase activity of the receptors was assayed by measuring incorporation of $^{32}P$ into the RR-src peptide as described under Experimental Procedures. The rate of kinase activity (picomoles per minute) is expressed as percent control.

allowed to equilibrate for 16 h at 202000*g* at 4 °C before use. The samples (0.1 mL) were layered on the top of each gradient, and centrifugation was performed for 4 h at 202000*g* at 4 °C. Twenty fractions of 240 μL were collected from the top down. The last fraction was removed after the tube was vortexed to free any receptors attached to the bottom of the tube.

## Results

*Effects of Various Polycations and Basic Proteins on IGF-I Receptor TPK Activity.* We have previously suggested that acidic domains in the insulin receptor may directly interact with polylysine (Fujita-Yamaguchi et al., 1989a). These domains are α633–645 (A-I), α695–707 (A-II), and β1268–1286 (A-III).[3] As expected from their overall sequence homology, IGF-I receptor also contains acidic domains similar to those of the insulin receptor: α682–694 (A-I), β737–753 (A-II), and β1253–1271 (A-III) (Ullrich et al., 1986). Among those, A-III of the insulin receptor is the domain which we suggested to be important for an interaction with polylysine (Fujita-Yamaguchi et al., 1989c).

In order to examine whether the two structurally related receptor TPKs respond differently to various basic polycations as suggested by others (Morrison et al., 1989), their effects on the TPK activity of the IGF-I receptor were measured under the same conditions as we have previously carried out with the insulin receptor (Fujita-Yamaguchi et al., 1989a). The results are summarized in Figure 1, in which the rate of TPK activity is expressed as percent of control. IGF-I receptor TPK was 3–7-fold-stimulated in the presence of polylysine and polyornithine. These results are in good agreement with those of the insulin receptor TPK (Fujita-Yamaguchi et al., 1989a). Protamine sulfate, however, stimulated the IGF-I receptor TPK more effectively than it did insulin receptor TPK.

*Effects of Polylysine and Polyarginine on Autophosphorylation and TPK Activities of the Insulin Receptor.* Kohanski reported that polyarginine stimulates insulin receptor autophosphorylation (Kohanski, 1989) while we previously observed that it inhibits insulin receptor TPK activity (Fujita-Yamaguchi et al., 1989a) and also found no effect on IGF-I receptor TPK activity (Figure 1). Therefore, the effects of both polylysine and polyarginine on autophosphorylation and TPK activities of the two receptors were closely compared.

Insulin receptor autophosphorylation was stimulated by either polylysine or polyarginine (autoradiograms are not shown, but representative data are seen in Figure 2B). Although both polycations stimulated autophosphorylation more effectively than insulin, polyarginine was far more effective than polylysine. Since this result is opposite to what we observed with exogenous substrate (RR-src peptide) phosphorylation (Fujita-Yamaguchi et al., 1989a), effects of polylysine on phosphorylation of both RR-src peptide and the β subunit were simultaneously measured from the same insulin receptor TPK assay mixtures (Figure 2A,B).[4] Polylysine stimulated the receptor kinase activity as judged by phosphorylation of RR-src peptide while polyarginine had no effect or was even slightly inhibitory at 1 μM (Figure 2A). This confirmed our previous observation (Fujita-Yamaguchi et al., 1989a). With respect to the β-subunit phosphorylation, the polyarginine-treated receptor, which had a very small effect on phosphorylation of RR-src peptide, was autophosphorylated to a much greater extent than the polylysine-treated receptor (Figure 2B). These results revealed that polyarginine greatly stimulates autophosphorylation with only minor changes in substrate

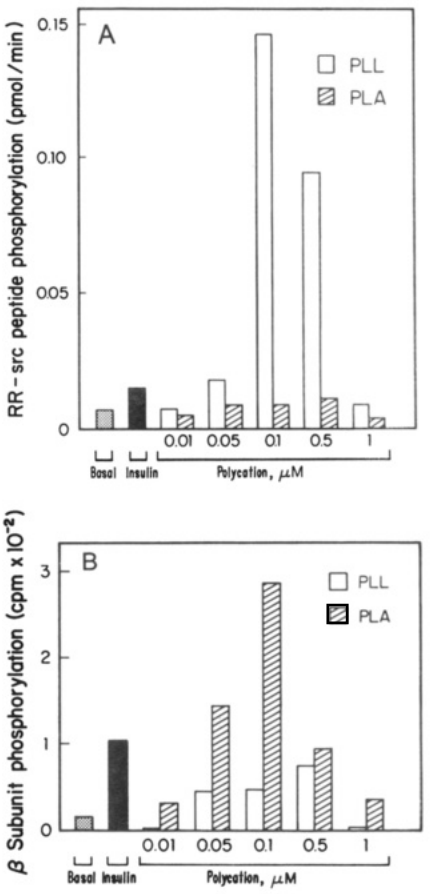


FIGURE 2: Differential effects of polylysine or polyarginine on kinase and autophosphorylation activities of the purified insulin receptor. Purified insulin receptor (~50 ng) was preincubated with the indicated concentrations of average $M_r$ = 41 000 polylysine [PLL (open bars)] or polyarginine [PLA (hatched bars)]. The receptors were subjected to the tyrosine kinase assay as described under Experimental Procedures. At the end of the phosphorylation reaction, half of the reaction mixture was used to measure $^{32}P$ incorporation into the RR-src peptide (A), and the other half of the same reaction mixtures was used to measure $^{32}P$ incorporation into the β subunit (B). As controls, effects of basal [no additions (stippled bars)] and 0.1 μM insulin (black bars) were included.

[3] Amino acid numbers from Ullrich et al. (1985) are used.

[4] Polycation concentrations for obtaining maximal stimulation of the receptor TPKs varied between different receptor preparations. This is most likely due to the difference in amounts of the polycation-sensitive receptor in assay mixtures. This peptide substrate has no effect on autophosphorylation of the insulin or IGF-I receptor β subunit.

phosphorylation. In contrast, polylysine seemingly stimulated both autophosphorylation and TPK activities with more stimulation of the latter (Figure 2A,B).

Concentration dependence for the effects of polylysine on RR-src phosphorylation activity of the insulin receptor revealed biphasic activation (Figure 2A). In order to examine whether inhibition of the RR-src phosphorylation observed in the presence of higher concentrations of polylysine is due to the interference of RR-src peptide binding to phosphocellulose papers by the polycation, the same assay mixtures as shown in Figure 2A were analyzed by paper electrophoresis (2000 V, 1 h, pH 3.5). The results were the same as those obtained by the phosphocellulose method, suggesting that the biphasic effect of polylysine on the RR-src phosphorylation activity is not an artifact.

Concentration dependence for the effects of polylysine and polyarginine on autophosphorylation was also biphasic (Figure 2B). In the presence of high concentrations of polycations, e.g., 200 $\mu$M, the $\beta$ subunit was not seen on SDS–PAGE gels when stained with silver (data not shown). This suggests that polylysine caused the formation of receptor aggregates which prevented the receptor from entering the polyacrylamide gel (see below). At a concentration of 500 $\mu$M polylysine, insulin receptor precipitates were visible.

*Effects of Polylysine and Polyarginine on Autophosphorylation and TPK Activities of the IGF-I Receptor.* Polylysine markedly stimulated IGF-I receptor autophosphorylation whereas polyarginine had a very small effect on the autophosphorylation (Figure 3A,B). Polylysine also stimulated RR-src peptide phosphorylation activity 9–10-fold at concentrations of 0.5 and 1 $\mu$M whereas polyarginine had little or an inhibitory effect on the TPK kinase activity (Figure 3C). Thus, in the case of IGF-I receptor TPK, effects of polylysine and polyarginine on autophosphorylation are correlated well with those on TPK activity.

The control experiments shown in Figure 3 indicated that although IGF-I stimulates autophosphorylation an average of 1.4-fold [(A) 1.6-fold, (B) 1.2-fold], the phosphorylation of RR-src peptide by the same receptor TPK is not significantly stimulated by IGF-I. Polylysine, however, can stimulate kinase activity ~10-fold (Figure 3C), suggesting that polylysine activates the IGF-I receptor TPK independently of IGF-I. The same situation has been observed with the insulin receptor TPK (e.g., Figure 2), in which insulin stimulation of autophosphorylation is better than that of RR-src peptide, ~5-fold vs ~2-fold. Previously, we have documented that polylysine can stimulate insulin receptor TPK which has lost insulin-stimulated kinase activity (Fujita-Yamaguchi et al., 1989).

*Effects of Different Molecular Sizes of Polylysine on Insulin and IGF-I Receptor TPKs.* Three polylysine polymers with average $M_r$ = 12000, 41000, and 289000 were effective in stimulating RR-src peptide phosphorylation by insulin receptor TPK (data not shown). Maximal stimulation achieved was ~10- to -20-fold for these three polylysines while insulin stimulated ~5-fold above the basal activity. The smallest polylysine that we examined (average $M_r$ = 3800) had a small but significant effect at a concentration of 0.5 mM. Concentrations required for half-maximal stimulation were 0.0042, 0.08, 1.5, and 250 $\mu$M for polylysine polymers with average $M_r$ = 289000, 41000, 12000, and 3800, respectively. The ratio of molecular weights of these polymers is 76:11:3:1 for average $M_r$ = 289000, 41000, 12000 and 3800. Thus, the effectiveness of polylysine on kinase activation appears to correlate with the length of the polymer except an average $M_r$ = 3800 polylysine. This polylysine is much less effective than

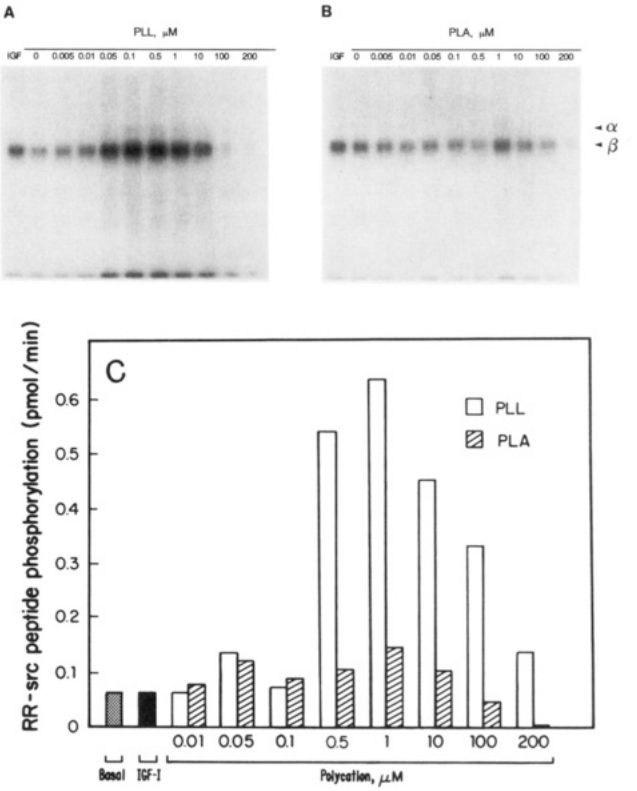


FIGURE 3: Effects of polylysine and polyarginine on autophosphorylation and kinase activities of the purified IGF-I receptor. Purified IGF-I receptor (~0.2 $\mu$g) was preincubated with the indicated concentrations of either average $M_r$ = 41000 polylysine or polyarginine. The receptors were subjected to the tyrosine kinase assay as described under Experimental Procedures. At the end of the phosphorylation reaction, half of the reaction mixture was used to examine $^{32}P$ incorporation into the $\beta$ subunit (A and B), and the other half of the same reaction mixtures was used to measure $^{32}P$ incorporation into the RR-src peptide (C). In (C), the results of polylysine [PLL (open bars)] and polyarginine [PLA (hatched bars)] are expressed as picomoles per minute. As controls, effects of basal [no additions (stippled bar)] and 0.1 mM IGF-I (black bar) were included.

an average $M_r$ = 12000 polylysine. The data indicate that a certain conformation may be required for the stimulation since the charge itself is not enough to activate the TPK; for instance, to obtain the same level of stimulation by a 500 $\mu$M sample of an average $M_r$ = 3800 polylysine, only a 0.2 $\mu$M sample of an average $M_r$ = 12000 polylysine was required, which means that the latter is 2500-fold more effective in the kinase activation although the difference in their average molecular weights (i.e., net charge) is 3-fold.

Similar results were obtained with IGF-I receptor (data not shown): Three polylysine polymers with average $M_r$ = 12000, 41000, and 289000 stimulated RR-src peptide phosphorylation by the IGF-I receptor TPK an average of ~10-fold whereas IGF-I activated the kinase activity ~2.5-fold.[5] Concentrations required for half-maximal stimulation of the kinase activity were 0.005, 0.05, 1.7 and 260 $\mu$M for polylysine polymers with average $M_r$ = 289000, 41000, 12000, and 3800, respectively.

*Effects of K-ras Basic Peptides on Insulin and IGF-I Receptor TPK and Autophosphorylation Activities.* The C-terminal 14 amino acid stretch of human K-ras is the most basic domain found among all proteins whose sequences are

[5] This particular IGF-I receptor preparation is more sensitive to stimulation by ligand or polylysine than the receptor preparation shown in Figure 3.

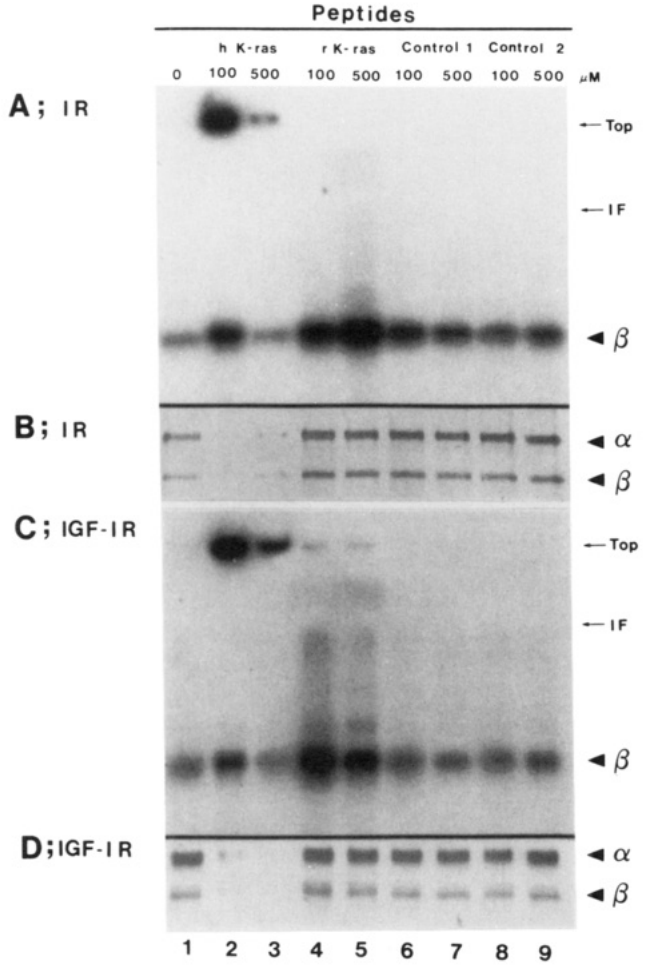


FIGURE 4: Effects of K-ras basic peptides and control peptides on insulin and IGF-I receptor autophosphorylation. Purified insulin and IGF-I receptors (~0.1 μg) were preincubated with 100 or 500 μM human K-ras peptide (h K-ras, lanes 2 and 3 ), rat K-ras peptide (r K-ras, lanes 4 and 5), or control peptides (1, CSSTSVDTSPEEHR, lanes 6 and 7; 2, CESEDSGKHNGSEYE, lanes 8 and 9). Autophosphorylation assays and SDS–PAGE under reducing conditions were performed as described under Experimental Procedures. (A) Insulin receptor, autoradiogram; (B) insulin receptor, silver-stained gel; (C) IGF-I receptor, autoradiogram; (D) IGF-I receptor, silver-stained gel. The top of SDS–PAGE gels and the interface (IF) between stacking and separating gels are indicated with arrows.

currently known. This domain contains eight lysines in which six lysines are consecutive and two lysines are near the lysine cluster. Rat K-ras sequence is similar to the human C-terminal domain except that two lysines are replaced with two arginines, in addition to minor differences near the C-terminus (see Experimental Procedures). We examined whether these naturally occurring protein domains can mimic the effect of polylysine on the receptor TPKs.

When the effects of human and rat K-ras peptides (0.1–500 μM) on the insulin receptor autophosphorylation together with those of insulin and polylysine were examined, both K-ras peptides significantly stimulated autophosphorylation of the insulin receptor β subunit with maximal stimulation at a concentration of ~100 μM (these data are not shown, but representative data are seen in Figure 4A, lanes 1–5). The maximal stimulation by 100 μM human K-ras basic peptide and by 100 μM rat K-ras basic peptide was 3.3-fold and 8.8-fold, respectively, whereas the stimulation by 0.1 μM insulin and 0.24 μM polylysine was 13-fold and 36-fold, respectively. Similarly, autophosphorylation of the IGF-I receptor β subunit appeared to be stimulated by the K-ras peptides (Figure 4C, lanes 1–5).

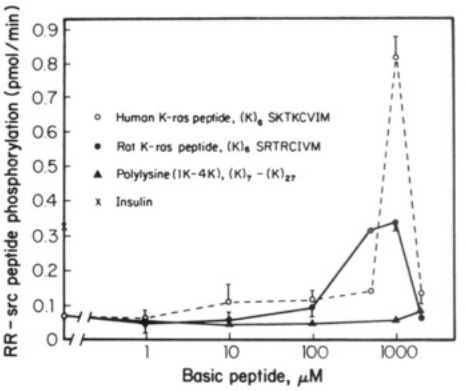


FIGURE 5: Effects of K-ras basic peptides on the kinase activity of the insulin receptor. Purified insulin receptor (~0.1 μg) was preincubated with the indicated concentrations of human (○) or rat (●) K-ras peptides, and RR-src peptide phosphorylation activity was measured. As controls, receptors treated with 0.1 μM insulin [(×) on the $y$ axis] or different concentrations of average $M_r$ = 3800 polylysine (▲) were included. The results shown are the average of three independent experiments.

Autophosphorylation of both insulin and IGF-I receptors was also performed in the presence of two control peptides containing one Cys residue but no basic amino acid stretches. The results shown in Figure 4 demonstrated that K-ras peptides, especially rat K-ras peptide, significantly stimulate autophosphorylation of the β subunits of both receptors whereas the control peptides had no effects. These data indicate that the effects of K-ras peptides are not due to the Cys residue, which may act as a reducing agent, but are most likely due to their unique basic domains. It should be noted that human K-ras peptide induced aggregation of both insulin and IGF-I receptors as seen in Figure 4, lanes 2 and 3, since silver staining (B and D) revealed very little β subunit in the gel. However, when $^{32}P$ incorporation into the β subunit per amount of β-subunit protein in lanes 2 and 3 was compared with those of the controls (lanes 6–9), the specific radioactivities of the β subunit treated with human K-ras peptide are clearly greater than those of the controls. In addition, $^{32}P$ incorporation into the aggregates remaining at the top of the gel as detected by autoradiography (A and B) was intensive, suggesting that human K-ras peptide is also effective in stimulating autophosphorylation of insulin and IGF-I receptors.

Effects of both K-ras peptides on RR-src phosphorylation activity of insulin receptor kinase as shown in Figure 5.[6] The human K-ras peptide at a concentration of 1000 μM stimulated the kinase activity more than twice as effectively as insulin whereas rat K-ras peptide at concentrations of 500 and 1000 μM stimulated the TPK to the same extent as insulin. In contrast, polylysine with average $M_r$ = 3800, which resembles K-ras peptides in terms of size, hardly stimulated the enzyme activity although it contains more residues of lysine than the K-ras peptides. The fact that the K-ras basic peptides are more effective in stimulating the insulin receptor TPK than lysine homopolymers may suggest that the K-ras domains contain a certain conformation which could effectively activate the TPK.

*Nondenaturing Gradient Gel Electrophoretic Analyses of IGF-I or Insulin Receptor TPKs Activated by Ligands and Polylysine.* Nondenaturing gradient gel electrophoresis was used next in order to analyze autophosphorylation activity and

[6] Effects of the K-ras-peptides on the IGF-I receptor TPK activity were examined 3 times. Unlike the insulin receptor TPK, the results were, however, inconsistent.

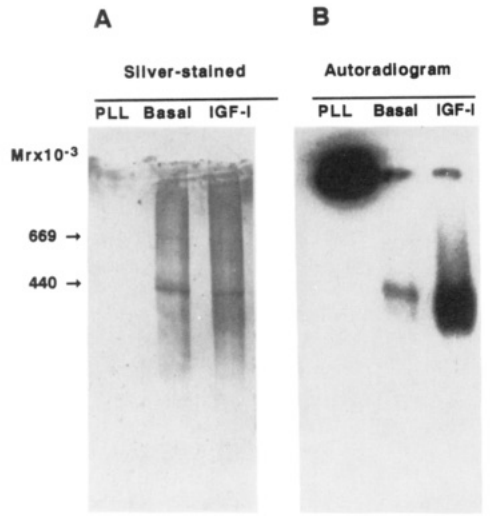


FIGURE 6: Nondenaturing gradient polyacrylamide gel electrophoresis on the IGF-I receptor treated with polylysine or IGF-I. Purified IGF-I receptor (~50 ng) was preincubated with 0.24 μM average $M_r$ = 41 000 polylysine (PLL), no additions (Basal), or 0.1 μM IGF-I (IGF-I). Autophosphorylation reaction and gel electrophoresis were performed as described under Experimental Procedures. The gel was stained with silver (A) and autoradiographed (B).

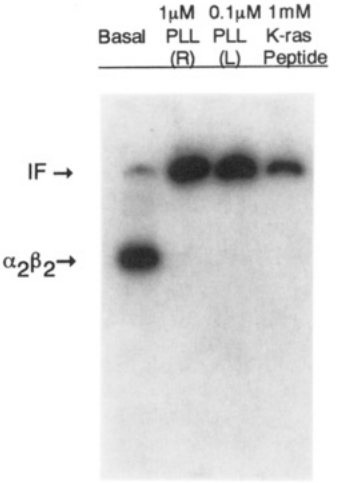


FIGURE 7: Electrophoretic mobility of the $^{32}$P-labeled IGF-I receptor before and after polylysine or basic peptide treatment. Purified IGF-I receptor (~0.2 μg) was autophosphorylated in the absence of polycations. The phosphorylated receptors were isolated by Sephadex G-50 chromatography (~2-mL column) in the presence of protease and phosphatase inhibitors and divided into four identical aliquots. Prior to SDS–PAGE under nonreducing conditions, the following were added: no additions (Basal), 1 μM average $M_r$ = 41 000 polylysine [PLL (R)], 0.1 μM average $M_r$ = 289 000 polylysine [PLL (L)], and 1 mM rat K-ras peptide. The position of $^{32}$P-labeled $\alpha_2\beta_2$ and the interface (IF) between the stacking and separating gels are indicated with arrows.

receptor size of the purified IGF-I receptor that had been treated with either IGF-I or polylysine. Since we used purified receptors, unlike previous studies which used crude receptors (Morrison et al., 1989; Kubar & Van Obberghen, 1989), we had the advantage of obtaining not only autoradiographical analysis but also protein staining patterns of the receptor. The silver-stained nondenatured gel (Figure 6A) revealed the protein band corresponding to the IGF-I receptor "monomer", $\alpha_2\beta_2$, for basal and IGF-I-treated receptors. However, no protein bands were observed with the polylysine-treated receptor. The autoradiogram of the same gel shows that IGF-I stimulated receptor autophosphorylation of an $\alpha_2\beta_2$ form whereas polylysine facilitated the aggregation of the receptor whose phosphorylation was extremely enhanced (Figure 6B). Although the protein band is not clearly seen, the receptor obviously remained at the top of the gel as the radioactivity remained at the top even after the silver staining. When the same sample was analyzed by SDS–PAGE, enhanced phosphorylation of the β subunit was detected as seen in Figure 3A,[5] suggesting that the high molecular weight radioactive band is highly aggregated receptors. Similar results were also obtained with the insulin receptor TPK (data not shown).

*Further Analyses on IGF-I Receptor Autophosphorylation and Aggregation.* To demonstrate that IGF-I receptor aggregation can be efficiently caused by saturated concentrations of polylysine polymers with average $M_r$ = 41 000 and 289 000 or rat K-ras peptide, the receptors which were first autophosphorylated and isolated by Sephadex G-50 chromatography were subjected to incubation with the polylysines or K-ras peptide and nonreducing SDS–PAGE analysis. Figure 7 shows that the phosphorylated receptors are mostly an $\alpha_2\beta_2$ form before the incubation. Polylysines facilitated aggregation of $\alpha_2\beta_2$ forms which are seen at the interface of the polyacrylamide gel. Unlike other experiments, the intensity of the receptor phosphorylation after incubation with polylysines remained the same as the basal rate since the autophosphorylation reaction did not take place in the presence of the receptor polycations. Rat K-ras peptide also facilitated receptor aggregation.

Finally, to support the results of nondenaturing gradient gel or nonreduced SDS–PAGE analysis which indicated receptor aggregation by polylysine or K-ras peptide, sucrose density gradient centrifugation as employed by Kohanski (1989) for the insulin receptor was performed. Figure 8 shows that the IGF-I receptors which were autophosphorylated in the presence of 1 μM average $M_r$ = 41 000 polylysine moved to the bottom of the tube as detected by both autoradiography (C) and silver staining (D). The radioactivity of the β subunit was greatly enhanced as compared with the control [no additions (A)]. The receptors in the control moved to the same position as the IGF-I binding peak (A). Since the receptors were distributed in approximately eight tubes, they were not visible after silver staining (B), in contrast to polylysine-induced receptor aggregates which were visible since they were concentrated in the bottom fraction (D).

## Discussion

In the present study, we first compared the TPK activities of two structually related receptors in the presence of the polycations polylysine and polyarginine. Throughout this study, the following special attention was given to accomplish each experiment: (i) the experiments were performed in the absence of IGF-I or insulin to observe only the direct effect of polycation on the receptor TPK; (ii) concentration-dependent curves were always measured; (iii) effects of both polylysine and polyarginine were measured in parallel using the same receptor preparation; and (iv) when necessary, effects of the polycation on autophorphorylation and RR-scr-peptide phosphorylation were measured in parallel using the same reaction mixture.

In summary, the work described here demonstrates (i) polylysine stimulates autophosphorylation and exogenous substrate phosphorylation avtivities of both IGF-I and insulin receptor TPKs, (ii) polyarginine affected both TPKs in a manner quite different from polylysine in that it stimulated insulin receptor autophosphorylation to a greater extent than polylysine did while it had a very small effect on the IGF-I receptor autophosphorylation and exogenous substrate phosphorylation activities of both receptor TPKs, (iii) K-ras pep-

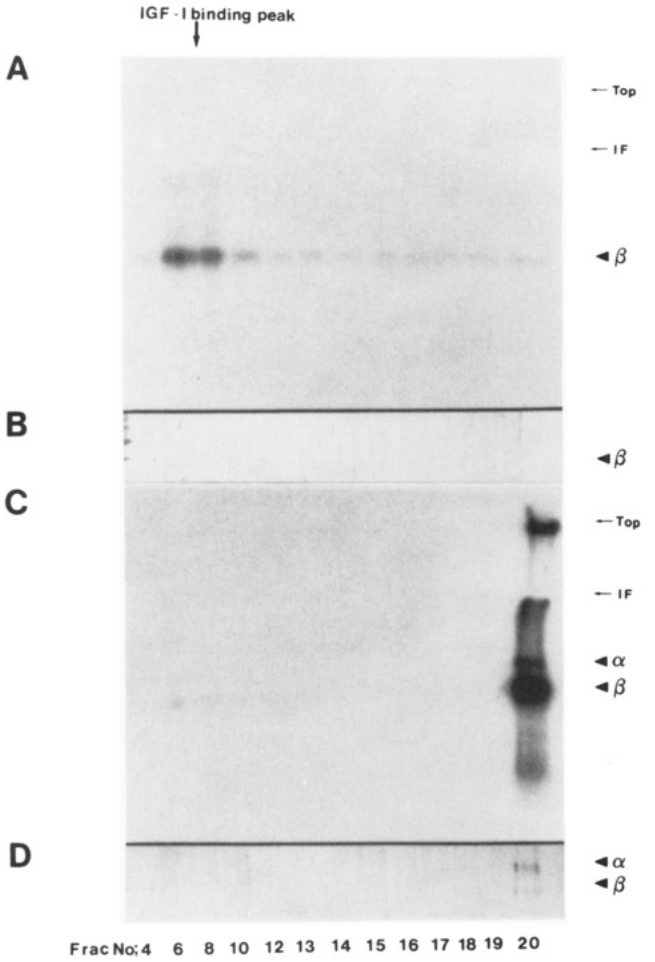


FIGURE 8: Aggregation of the IGF-I receptors and activation of β-subunit phosphorylation caused by polylysine as judged by sucrose density gradient centrifugation and SDS–PAGE. The purified IGF-I receptor (~0.1 μg) was preincubated with buffer or 1 μM average $M_r$ = 41 000 polylysine for 30 min at 25 °C. Autophosphorylation reactions were then carried out as described under Experimental Procedures. The reaction mixtures were diluted with 50 mM Tris-HCl buffer, pH 7.4, to give a volume of 0.1 mL and layered onto 4.7-mL gradients as described under Experimental Procedures. After centrifugation and collection of fractions of 240 μL each, 10-μL aliquots of the fractions as indicated were subjected to SDS–PAGE under reducing conditions. The gels were silver-stained (B and D), dried, and autoradiographed (A and C). (A and B) Basal (no additions); (C and D) 1 μM average $M_r$ = 41 000 polylysine. The top and interface (IF) of SDS–PAGE gels are indicated with arrows.

tides, domains of natural proteins containing a polylysine-like sequence, had similar effects on the two receptor TPKs to those of polycations, and (iv) receptor aggregation caused by polycations or K-ras peptides may be a potential mechanism for kinase activation.

As expected from their homologous primary structures (Ullrich et al., 1986), we consistently observed, similarly to the insulin receptor TPK, that IGF-I receptor TPK activity was stimulated in the presence of polylysine, polyornithine, and protamine, as judged by RR-src peptide phosphorylation. The reasons why Morrison et al. (1989) were unable to observe the activation of IGF-I receptor TPK are not clear. The major difference between their and our experiments is that they used partially purified placental receptors which contained both insulin and IGF-I receptors whereas we used highly purified insulin and IGF-I receptors. It is interesting to see that they did not detect insulin receptor TPK activation by polylysine in the absence of insulin although we showed a marked activation of insulin receptor TPK in the absence of insulin (Fujita-Yamaguchi et al., 1989a; this study). This may be due to extremely low TPK activity in their preparations. In addition, effects of other protein contamination in the partially purified receptor preparations may have caused further complications. We have previously observed with partially purified WGA–Sepharose eluates that basic proteins stimulate Ser/Thr kinases which phosphorylate the β subunit as well as other proteins (Sacks et al., 1989a). Kohanski (1989) also reported that there are both quantitative and qualitative differences in the effects of 40-kDa polyarginine on autophosphorylation of partially purified insulin receptors as compared to the purified receptor.

In previous reports, we suggested that polylysine directly interacts with the insulin receptor β subunit, thereby inducing activation of the TPK (Sacks et al., 1989a; Fujita-Yamaguchi et al., 1989c). Substrate phosphorylation by prephosphorylated receptors was more effectively activated by polylysine than nonphosphorylated receptors, which also indicated possible direct interactions between the polycation and insulin receptor β subunit (Sacks et al., 1989a). In this study, we made several observations which indicate the formation of polylysine–receptor complexes during activation of the TPK: (i) Nondenaturing gel electrophoretic analyses revealed that polylysine appears to have caused a shift in an equilibrium between $\alpha_2\beta_2$ monomers and aggregates of the IGF-I or insulin receptors under the conditions where we observed TPK activation. In contrast, IGF-I or insulin appears to have activated the monomeric forms as well as aggregate forms without changing their ratios. (ii) The concentration effects of polylysines were biphasic. This indicates a stoichiometric interaction of the two molecules; that is, as the aggregation exceeds more than an optimal condition, at higher concentrations of polylysine (>100 μM) TPK activation can no longer be observed. The receptors seem to be precipitated at >100 μM polylysine as we have clearly seen the precipitate at 500 μM polylysine (silver-stained gel not shown). Inhibition of the TPK activity observed at >100 μM polylysine is likely due to "salting out" effects of polylysine. The receptor precipitates did not dissolve in the SDS–PAGE sample buffer, which suggests that the receptors were insoluble and therefore no longer able to exhibit TPK activity. (iii) The effectiveness of polylysine on TPK activation appears to be proportional to the length of the polymer except for an average $M_r$ = 3800 polylysine. This suggests that optimal ratios of polylysine units and TPKs are required for TPK activation.

We previously reported than an aggregated form of insulin receptors is a much more active TPK as judged by radiation inactivation analyses (Fujita-Yamaguchi et al., 1989b). Although an exact mechanism of this activation is not known, we have suggested that a certain conformation of the receptor caused by the aggregation of 8–10 $\alpha_2\beta_2$ monomers is required for high TPK activity. These aggregated forms, $(\alpha_2\beta_2)_{8-10}$, exist in out purified insulin receptor preparations although the estimated protein amount is ~6% (Fujita-Yamaguchi et al., 1989b). The fact that polylysine-induced TPK activation was shown to be biphasic while the aggregation process seems to continue as the concentration of polylysine increases is consistent with our previous observation that the receptor aggregate of $(\alpha_2\beta_2)_{8-10}$ is a highly active kinase. Further investigation is required, however, to understand the nature of receptor aggregates. Especially, it is important to find whether the receptor aggregation process involves disulfide exchanges or strong noncovalent interactions.

With respect to insulin receptor aggregation, Kubar and Van Obberghen showed that receptor forms with ~1200, ~950, and ~580 kDa were resolved by nondenaturing gel electro-

phoresis and that the highest ~1200-kDa oligomer exhibits the most pronounced autophosphorylation (Kubar & Van Obberghen, 1989). Their observation is consistent with our previous and present data indicating that receptor aggregates possess extremely high TPK activity. Kohanski (1989) also showed by sucrose density gradient centrifugation that 40-kDa polyarginine induced aggregation of both prephosphorylated and non-prephosphorylated purified insulin receptors. He suggested that polycation-stimulated receptor autophosphorylation is linked to a lower apparent $K_m$ for ATP and that substrate phosphorylation requires aggregation. Our results are generally in good agreement with those of his in that we both observed insulin receptor aggregation and TPK activation in the absence of insulin. However, he did not show the biphasic effects of polylysines which are intrinsic to the nature of polycation-stimulated TPK activation as we have shown.

While this paper was in preparation, we learned that a similar study to ours was in press (Biener & Zick, 1990). Biener and Zick observed the following: (i) Polylysine was able to activate the IGF-I receptor TPK prepared from rod outer segment membranes. (ii) The stimulatory potential of polylysine is proportional to its chain length. (iii) Polylysine poorly activates insulin receptor TPK immobilized on either wheat germ agglutinin–, insulin–, or polylysine–agarose columns, indicating that the polylysine activation involves induction of conformational changes. (iv) The effect of polylysine on autophosphorylation and TPK activities of the insulin receptor is biphasic. Their conclusion is similar to ours that polylysine-induced receptor aggregation could be a potential mechanism of kinase activation. However, as they also indicated, we cannot rule out the possibility that polylysine-induced aggregation is only a coincidental effect on the receptors.

The polycation-induced receptor TPK activation as described is rather an in vitro phenomenon. Polycations are obviously useful tools to compare activities of purified enzymes. However, their biological significance is difficult to address since polycations are known to have a variety of effects on cells and enzymes (Ham & McKeeham, 1979; Gatica et al., 1987; Pelech & Cohen, 1985; Brunati et al., 1985; Yanagita et al., 1987). Hence, we examined whether or not natural proteins containing polylysine-like domains can mimic the effect of polylysine. We found that the peptides corresponding to human and rat K-ras C-terminal 14 amino acid residues have stimulatory effects on autophosphorylation and RR-src peptide phosphorylation activities of the insulin receptor TPK. The effect of human K-ras peptide on insulin receptor autophosphorylation is consistent with the previous observation by Sacks et al. (1989b). Our present studies were extended to examine (i) effects of two related K-ras peptides which differ in three amino acid sequences as described under Experimental Procedures, (ii) both autophosphorylation and RR-src peptide phosphorylation activities, (iii) both insulin and IGF-I receptor TPKs, and (iv) molecular size analyzed by SDS–PAGE under reducing or nonreducing conditions. The stimulatory effects of the K-ras peptides were not as great as those of polycations. However, when compared with an average $M_r$ = 3800 polylysine, the K-ras peptides containing a fewer number of basic residues than this polylysine showed more stimulatory effects on the insulin receptor TPK (Figure 7). This evidence indicates that K-ras domains may have a conformation which is required for an efficient interaction with the receptor TPK and that endogenous proteins in cells such as K-ras proteins may modulate receptor TPK activity. SDS–PAGE analyses revealed that the K-ras peptides also promoted aggregation of IGF-I and insulin receptors, suggesting that aggregation may also be the mechanism for the stimulatory effects of K-ras peptides.

## ACKNOWLEDGMENTS

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**Registry No.** IGF-I, 67763-96-6; IGF-I receptor kinase, 103843-29-4; PLL, 25104-18-1; PLA, 24937-47-1; PLO, 24937-49-3; RR-src peptide, 81156-93-6; insulin, 9004-10-8; insulin receptor kinase, 88201-45-0.

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# Fluorescence Assay for Phospholipid Membrane Asymmetry†

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ABSTRACT: Highly fluorescent 7-nitro-2,1,3-benzoxadiazol-4-yl-lipid (NBD-lipid) analogues are widely used to examine lipid transport and membrane structure. We have developed a method for chemically modifying NBD-labeled lipids in both artificial and biological membranes. This was achieved by treating fluorescently labeled membranes with dithionite ($S_2O_4^{-2}$). When small unilamellar vesicles containing NBD-labeled phospholipids were reacted with dithionite, only the fluorescent lipid located on the outer leaflet of the vesicles' bilayer was reduced. Seven different NBD-lipid analogues, including a fluorescent sterol, were reduced by treatment with dithionite to nonfluorescent 7-amino-2,1,3-benzoxadiazol-4-yl-lipid derivatives. To assess the feasibility of using this reagent in biological systems, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine was inserted into the outer leaflet of the plasma membrane of CHO-K1 cells. Subsequent incubation of these cells with a nontoxic concentration of dithionite resulted in the complete loss of fluorescence from the plasma membrane. In contrast, when cells were permitted to endocytose some of their fluorescently labeled plasma membrane and then treated with dithionite, fluorescence at the plasma membrane was eliminated, while intracellular labeling was not affected. These data suggest that dithionite reacts with NBD-labeled lipids in the outer leaflet of membrane bilayers, producing nonfluorescent derivatives. We demonstrate how reduction of NBD-lipids with dithionite can be used to prepare asymmetrically labeled liposomes and to measure transverse-membrane asymmetry in vesicles. This method should be useful in many biochemical investigations, including the measurement of phospholipid translocase activity.

Phospholipids have an asymmetric transbilayer distribution across the plasma membranes of a variety of cell types (Op den Kamp, 1977). Membrane phospholipid asymmetry has been most vigorously studied in human erythrocytes. Aminophospholipids are located primarily in the inner leaflet of erythrocyte membranes while phosphatidylcholine and sphingomyelin are found primarily in the outer leaflet (Bretscher, 1972; Gordesky & Marinetti 1973; Verkleij et al., 1973). Maintenance of phospholipid asymmetry in erythrocytes is of physiological significance. For example, the appearance of phosphatidylserine on the outer leaflet of the membrane may be a signal for erythrocyte removal from the circulation by the reticuloendothelial system (Tanaka & Schroit, 1983; Schroit et al., 1985). Formation and maintenance of phospholipid asymmetry in erythrocytes probably involves an ATP-dependent *N*-ethylmaleimide-sensitive transport protein called aminophospholipid translocase (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986). Aminophospholipid translocase activity has been demonstrated in several other cell types, including platelets (Sune et al., 1987), lymphocytes (Zachowski et al., 1987), and fibroblasts (Sleight & Pagano, 1985; Martin & Pagano 1987). This activity has also been observed in synaptosomes (Zachowski et al., 1990) and chromaffin granules (Zachowski et al., 1989).

The most successful methods for monitoring aminophospholipid translocase activity require first labeling the outer leaflet of the test membrane with either a spin-labeled or fluorescent phospholipid analogue (Morrot et al., 1989; Connor & Schroit, 1988). The analogue is then allowed to be acted upon by the translocase for a specific time. To remove any analogue that remains at the outer leaflet, the cells are incubated with bovine serum albumin (BSA).[1] After separation

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