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Control of Insulin Receptor Autophosphorylation by Polypeptide Substrates: Inhibition and Stimulation by Interaction with the Catalytic Subunit†

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ABSTRACT: Autophosphorylation of purified insulin receptor, in the absence of insulin, was stimulated by selected polypeptide substrates. In the presence of 1 μM insulin these peptides inhibited autophosphorylation. Stimulation was observed with reduced [*S*-(carboxamidomethyl)cysteinyl]lysozyme (RCAM-lysozyme) and three peptides generated by CNBr cleavage, V8 proteinase digestion, and/or chemical modification. We also generated two peptide substrates from RCAM-lysozyme which did not stimulate receptor autophosphorylation and were very weak inhibitors. As a control peptide, the simple substrate angiotensin inhibited receptor autophosphorylation in the absence or presence of insulin. However, stimulatory peptide, but not insulin, significantly shifted the concentration dependence for inhibition by angiotensin. The stimulatory peptides also increased autophosphorylation of the cloned cytoplasmic domain of the kinase [R-BIRK; Villalba, M., Wente, S. R., Russell, D. S., Ahn, J., Reichelderfer, C. F., & Rosen, O. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7848]. Therefore, stimulation occurs by interaction with the cytoplasmic process of the β-subunit and not through interaction with the insulin binding α-subunit of the native receptor. Autophosphorylation was analyzed by mapping ^{32}P-labeled tryptic phosphopeptides from the β-subunit and from R-BIRK. Nearly identical phosphopeptide maps were found, comparing first, basal R-BIRK and basal native receptor, second, peptide- and insulin-stimulated native receptor, and third, peptide-stimulated R-BIRK and insulin-stimulated native receptor. Therefore, R-BIRK functions as a basal-state enzyme and can be stimulated in an insulin-like manner. On the basis of these observations, stimulation by insulin and by peptides yields similar functional results, but by apparently different mechanisms.

The insulin receptor is a protein-tyrosine kinase with a native $\alpha_2\beta_2$ disulfide-linked structure. The α-subunit binds insulin, and the β-subunit bears the protein kinase activity. Insulin stimulates autophosphorylation of the β-subunit in intact cells (Klein et al., 1986; Kohanski, et al., 1986; Pang et al., 1985). After insulin-stimulated autophosphorylation in vitro, exogenously added polypeptide substrates are phosphorylated at an increased rate, compared to the aporeceptor (Rosen et al., 1983; Herrera & Rosen, 1986; Tornqvist & Avruch, 1988).

Autophosphorylation is an intrinsic reaction of the insulin receptor. A large body of evidence supports a biological role for autophosphorylation, and possibly for substrate phosphorylation as well [these properties are reviewed comprehensively by Rosen (1987)]. There is also evidence that challenges this view (Simpson & Hedo, 1984; Debant et al., 1989; Hawley et al., 1989; Baron et al., 1989; Soos et al., 1989) or suggests alternative pathways not dependent on protein kinase activity (Kelly et al., 1986; Saltiel & Cuatrecassas, 1986; Saltiel et al., 1986; Witter et al., 1988).

A major issue concerning the autophosphorylation reaction is the mechanism of insulin activation. To delineate a formal

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kinetic mechanism, inhibitors of enzyme-catalyzed reactions are necessary tools. In the case of protein kinase autophosphorylation, polypeptide substrates (Bertics & Gill, 1985; Shoelson et al., 1988; Kwok et al., 1986; Morrison & Pessin, 1987) and/or their nonphosphorylatable analogues (Shoelson et al., 1989) have been the inhibitors of choice. With the high-affinity substrate RCAM-lysozyme[1] (Kohanski & Lane, 1986), we planned to study inhibition of the activated versus the basal state, i.e., in the presence versus the absence of insulin, respectively. We report here anomalous patterns in the inhibition of autophosphorylation by RCAM-lysozyme. The most surprising effect was stimulation of basal-state autophosphorylation by this substrate. Through a strategy of chemical modifications and enzymatic cleavages of RCAM-lysozyme, we fashioned polypeptides with variable inhibitory and stimulatory potencies. Our evidence supports a novel regulatory subdomain, not previously recognized, on the protein kinase subunit of the insulin receptor.

Experimental Procedures

Materials. [^{32}P]Orthophosphate was obtained from ICN Radiochemicals; [γ-^{32}P]ATP was synthesized following the method of Walseth and Johnson (1979) and purified according to Palmer and Avruch (1981). Serum and cell culture reagents were from Gibco; tetranitromethane was from Aldrich; hen egg white lysozyme, dithiothreitol, HEPES, and all enymes were from Boehringer/Mannheim; SDS, CNBr, and HPLC reagents were from Pierce; porcine insulin was from Eli Lilly Co. All other chemicals were reagent or electrophoresis grade.

Preparation of RCAM-lysozyme Fragments. Reduced [*S*-(carboxamidomethyl)cysteinyl]lysozyme (RCAM-lysozyme) was prepared as described previously (Kohanski & Lane, 1986). Briefly, 1 g of lysozyme was dissolved in 50 mL of 6 M guanidine hydrochloride, 0.2 M Tris-HCl, and 3 mM EDTA, pH 8.6, under nitrogen sparge. Dithiothreitol (10 mmol) was added; the mixture was heated at 60 °C for 1 h and then cooled to room temperature. Iodoacetamide (4.07 g) was added, and after 15 min at room temperature, 0.3 g of dithiothreitol was added to quench the reaction. RCAM-lysozyme was isolated by gel permeation chromatography on Sephadex G-25 (5 × 54 cm), developed by descending flow in 30% acetic acid (v/v). The product was lyophilized and stored desiccated at −20 °C.

The polypeptide substrate V8-3 (residues 36–129) and the fragment V8-2 (residues 8–35) were prepared by enzymatic digestion of 150 mg of RCAM-lysozyme with proteinase EndoGlu-C (V8 proteinase). RCAM-lysozyme at 5 mg/mL in 50 mM ammonium acetate, pH 4.0, was digested at 30 °C for 5 h with 2.2 mg of EndoGlu-C proteinase. Peptides were separated from the lyophilized digest by gel permeation chromatography on Bio-Gel P-60 (2.5 × 142 cm; descending flow) connected directly to Bio-Gel P-30 (2.5 × 202 cm; ascending flow) developed in 0.1% TFA (w/v)–10% acetonitrile (v/v), at a flow rate of 6 mL/h. The distribution of peptides in the elution profile, monitored at 280 and 220 nm, was assessed by SDS–PAGE according to the method of Swank and Munkres (1971). Fractions containing V8-3 (≥99% purity) were pooled, lyophilized, and stored desiccated at −20 °C. The yield of V8-3 was 78%.

Nitrosylation of V8-3 was performed according to the method of Sokolovsky et al. (1966): V8-3 was dissolved at 1 mg/mL in 0.1 M HEPES, 1 mM EDTA, and 2 M guanidine hydrochloride, pH 8.05. Two aliquots of 0.84 M tetranitromethane in 95% ethanol were added in 30-fold molar excess at 1.5-h intervals, and the pH was maintained near 8.0 with NaOH. Nitrotyrosyl-V8-3 was recovered by chromatography on Bio-Gel P-2 (1.5 × 30 cm) developed in 0.1 M ammonium acetate, pH 7, and lyophilized. Aminotyrosyl-V8-3 was prepared from nitrotyrosyl-V8-3 by the method of Sokolovsky et al. (1967), and the peptide was recovered from the reaction as described above.

Maleyl-V8-3 was prepared by addition of solid maleic anhydride to 5 mg of V8-3 dissolved in 3 M guanidine hydrochloride, 50 mM HEPES, and 1 mM EDTA, pH 8.5 (Butler & Hartley, 1972). The pH was maintained at 8.5–9.0 with additions of NaOH. The product was dialyzed against water and lyophilized.

The substrate VC-1 (residues 36–105) was prepared by CNBr cleavage of V8-3, according to the procedure of Gross (1967). V8-3 was dissolved at 3 mg/mL in 70% formic acid, under nitrogen. Cleavage was for 24 h at room temperature, with CNBr:peptide at 1:20 (w/w). VC-1 (17 mg, 39% yield) was purified from the lyophilized mixture by chromatography on Bio-Gel P-30 (2.5 × 202 cm; descending flow), directly into Bio-Gel P-10 (2.5 × 95 cm; ascending flow), developed in 0.1% trifluoroacetic acid (w/v)–10% acetonitrile (v/v). Purity of VC-1 in the eluate was determined by SDS–PAGE, as above.

Cyanogen bromide cleavage of RCAM-lysozyme was done as described above for the generation of VC-1. Purification of the fragments was by gel permeation chromatography, as above. By this procedure we obtained pure CB-2 in 19% yield and pure CB-3 in 78% yield.

Purification of the Insulin Receptor. Insulin receptor from mouse 3T3-L1 adipocytes and the cloned human insulin receptor, expressed in NIH 3T3 cells (Whittaker et al., 1987), were each purified by the same procedure (Kohanski & Lane, 1985). The purified receptor, eluted from the avidin–Sepharose column in complex with $N\alpha^{B1}$-biotinyl(ϵ-aminocaproyl)insulin, was freed of the insulin derivative by readsorption to 100–150 μL of wheat germ agglutinin–Sepharose, washed for 2 days with 50 mM HEPES–1 mM EDTA, pH 6.9, and eluted in this buffer at pH 8.5, containing 0.3 M *N*-acetylglucosamine. The soluble cytoplasmic domain of the insulin receptor expressed in Sf9 insect cells (Villalba et al., 1989) was the generous gift of Ora Rosen.

Assay of Protein Phosphorylation. The protein kinase—either the native $\alpha_2\beta_2$ receptor or the cytoplasmic domain of the kinase subunit—was used in its purified form only. Stock solutions of purified polypeptides were prepared in 50 mM HEPES, pH 6.9, and stored at −20 °C. Where present, insulin was mixed with the receptor prior to the assay, to give a final concentration of 1 μM. All assay mixtures were equilibrated for 15–20 min at room temperature before the reaction was begun.

The assays were performed at room temperature in 50 mM HEPES–0.06% Triton X-100 (w/v), pH 6.9. All reactions were initiated by the addition of 100–200 μM [γ-^{32}P]ATP plus 50 mM $Mn(CH_3CO_2)_2$, to give final concentrations of 10 or 20 μM and 5 mM, respectively.[2] The reactions were quenched

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; R-BIRK, the cytoplasmic kinase domain of the human insulin receptor; RCAM-lysozyme, reduced [*S*-(carboxamidomethylcysteine)]lysozyme; SDS, sodium dodecyl sulfate; CB-2, CB-3, VC-1, V8-2, and V8-3, peptides derived from RCAM-lysozyme, as defined in the text.

[2] Reactions for 5 min at 20 μM [γ-^{32}P]ATP yield ≈ 40% and 5% of end point, in the presence and absence of 1 μM insulin, respectively. The end point of receptor autophosphorylation was determined at 0.5 mM [γ-^{32}P]ATP, giving approximately 3 mol of phosphate incorporated per mole of insulin binding sites (R. A. Kohanski, unpublished observations).

after times indicated in the figures, by the addition of concentrated sample buffer, giving final concentrations of 2% SDS (w/v), 20 mM EDTA, and 20 mM dithiothreitol; samples were not heated. The ^{32}P-labeled β-subunit of the insulin receptor and ^{32}P-labeled substrate polypeptides were resolved by SDS-PAGE according to the method of Laemmli (1970). Two-phase slab gels were used: the upper phase was 10% T and 2.5% C, and the lower phase was 15% T and 4% C, retaining the β-subunit and polypeptide, respectively. Assays for the soluble kinase domain of the receptor (R-BIRK) were done under the above assay conditions but included bovine serum albumin at 0.5 mg/mL and 5 mM dithiothreitol; neither altered the results. The ^{32}P-labeled phosphoproteins generated in these reactions were resolved in single-phase gels: 15% T and 2.5% C.

Gel segments containing the ^{32}P-labeled phosphoproteins were excised after alignment of the dried gel with the autoradiograms. Background radioactivity in the gels was determined from segments near the phosphoproteins. All gel segments were of uniform size for each phosphoprotein. The segments were counted in 6 mL of Liquiscint, with a Beckman liquid scintillation counter, or were counted without scintillant but were first wetted with 20 μL of water to reduce artifactual counts from static.

Where we report a $K_{M,app}$ for a polypeptide substrate, that parameter was determined as follows: Purified insulin receptor was first autophosphorylated for 30 min at 200 μM [γ-^{32}P]-ATP–5 mM $Mn(CH_3CO_2)_2$. Peptides were added to give concentrations from 1 to 100 μM and a final [γ-^{32}P]ATP concentration of 20 μM. This reaction was quenched after 5 min, and radiolabeled peptides were isolated by SDS–PAGE. Michaelis constants were determined from Lineweaver–Burk plots.

^{32}P-Labeled Phosphopeptides of the β-Subunit. The autophosphorylation reactions were performed under conditions given in the figure legends. The ^{32}P-labeled β-subunit, or the soluble kinase domain, was isolated by SDS–PAGE. The appropriate segments were excised from wet, unfixed gels. These were soaked in water (2 × 50 mL) and once in 50 mL of 0.05 M *N*-ethylmorpholineacetic acid, pH 8.6 (1 h each). Digestion with 0.1 mg of trypsin in 1.0 mL of the last buffer was for 14–16 h at room temperature. ^{32}P-Labeled phosphopeptides were separated from the gel residue by centrifugation through 0.2 μm pore size Ultrafree microcentrifuge tubes (Millipore). The gel residue was washed with 200 μL of water, and the pooled filtrates were lyophilized. Each lyophilized sample was dissolved in 300 μL of water and relyophilized. The recoveries of radioactivity were ≥85%.

Each sample was dissolved in 0.15% trifluoroacetic acid in water (w/v). The trypic ^{32}P-labeled phosphopeptides were resolved by high-performance liquid chromatography. The chromatograph was a Hewlett-Packard System 1090 with an automated sample injector. The column was a Spherisorb C8 column with 3-μm particles (2 × 150 mm). Radioactivity in the eluate was detected with a Radiomatic Flow-one Beta continuous-flow radiation detector fitted with a 300-μL flow cell. The detector was set to update data at 10- or 15-s intervals.

The solvent system to develop the chromatograms was as follows: (A) 0.15% trifluoroacetic acid in water (w/v); (B) 0.1% phosphoric acid (85% stock) in acetonitrile (w/v). The initial solvent composition was 1% B. The percent solvent B was varied as follows: 0–10 min, 1%–2% B; 10–20 min, 2%–8.5% B; 20–35 min, constant at 8.5% B; 35–65 min, 8.5%–20.5% B; 65–70 min, constant at 20.5% B. The column was equilibrated at 1% B for 28 min between injections, which were made at 100-min intervals. The flow rate was 0.25 mL/min.

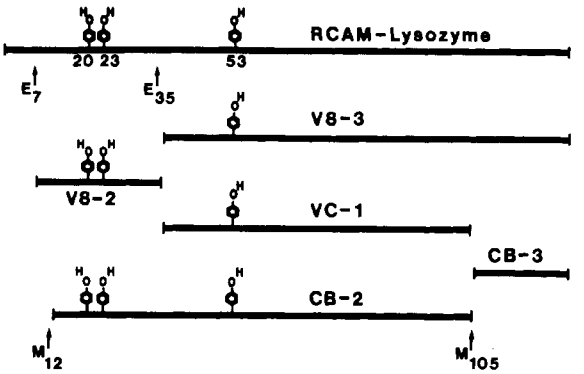

FIGURE 1: RCAM-lysozyme and its derivatives. There are three tyrosines in the polypeptide substrate RCAM-lysozyme (129 amino acids, 14.8 kDa). Cleavage with V8 proteinase at Glu-7 and Glu-35 produced three fragments: V8-1 (residues 1–7, not shown), which had no effect on receptor autophosphorylation; V8-2 (8–35), which was an inhibitor of autophosphorylation but was *not* a substrate; and V8-3 (36–129, 10.8 kDa), which included the single site of substrate phosphorylation at Tyr-53. Chemical modifications of V8-3 are described in the text. Cleavage of V8-3 with cyanogen bromide at Met-105 gave the substrate VC-1 (36–105, 8.1 kDa) and the inhibitor CB-3 (106–129). Direct CNBr cleavage of RCAM-lysozyme at Met-12 and Met-105 produced the substrate CB-2 (13–105, 10.7 kDa) and the inhibitor CB-3.

RESULTS

The Substrate Phosphorylation Site of RCAM-lysozyme. The polypeptide substrate RCAM-lysozyme and several fragments used in these studies are shown schematically in Figure 1. This substrate is phosphorylated exclusively on tyrosine by the purified insulin receptor (Kohanski & Lane, 1986). Several fragments shown in Figure 1 also serve as substrates for the insulin receptor protein (tyrosine) kinase: These were V8-3, its subfragment VC-1, and CB-2. In addition, maleyl-RCAM-lysozyme and maleyl-V8-3 were also substrates for the insulin receptor. However, the peptides V8-2, which includes Tyr-20 and Tyr-23, and CB-3, which has no tyrosine (see Figure 1), were *not* phosphorylated by the insulin receptor over the concentration range 0.01–500 μM, under any reaction conditions where phosphorylation of the other peptides occurred.

These observations indicated that Tyr-53 was the exclusive phosphorylation site on the substrate RCAM-lysozyme, since Tyr-53 was the only potential site shared by all the phosphorylatable peptide substrates. This was confirmed by tryptic digestion of the various phosphorylated peptides and separation of the products by HPLC (Figure 2). We detected a single ^{32}P-labeled phosphopeptide from tryptic digestion of RCAM-lysozyme and V8-3 in 93 ± 5% yield of injected radioactivity (shown in Figure 2) and also from maleyl-RCAM-lysozyme, VC-1, and CB-2 (not shown, although the results were identical with those presented). This result was repeatedly and consistently found with different concentrations of ATP up to 100 μM, reaction times up to 2 h, and various solvents, elutive gradients, and reversed-phase HPLC columns.

Finally, nitrosylation of Tyr-53 in the substrate V8-3 prevented its phosphorylation, over the concentration range 0.01–200 μM. Two likely explanations for this are, first, the nitro group adds enough bulk to impede entry of the tyrosyl group into the active site (see below), and second, nitrosylation lowers the pK' of the phenolic hydroxyl to ≈7 [Sokolovsky et al. (1967) and confirmed by spectrophotometric titration—not

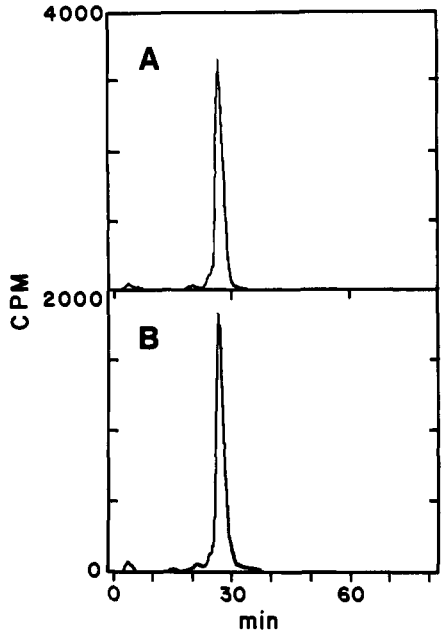

FIGURE 2: Analysis of the substrate phosphorylation site by tryptic ^{32}P-labeled phosphopeptide mapping. Substrate phosphorylation was done under the following conditions: Purified insulin receptor was incubated with 1 μM insulin, 10 μM [γ-^{32}P]ATP (195 cpm/fmol), and 5 mM $Mn(CH_3CO_2)_2$, pH 6.9, for 20 min. This preactivated receptor was then mixed with polypeptide substrates, each at 10 μM final concentration; these reactions were supplemented with [γ-^{32}P]ATP and $Mn(CH_3CO_2)_2$ to maintain their respective concentrations. The substrate phosphorylation reactions were quenched after 60 min and the products separated by SDS–PAGE (15% T, 2% C). Conditions for digestion with trypsin and chromatography are given in the text. The substrates phosphorylated were (panel A) V8-3 and (panel B) RCAM-lysozyme; not shown are VC-1, CB-2, and maleyl-V8-3. Additional data are given under Results.

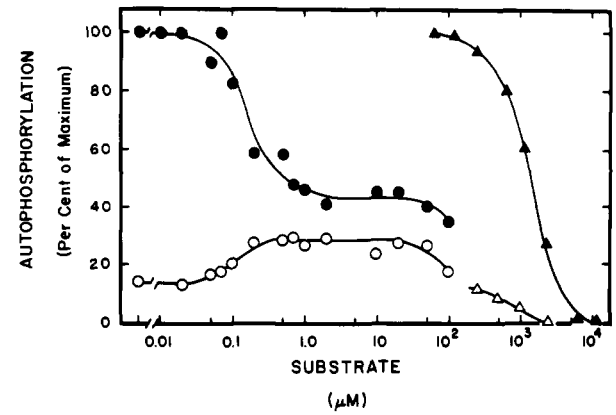

FIGURE 3: Polypeptide substrate-dependent changes in receptor autophosphorylation. Native insulin receptor from mouse 3T3-L1 adipocytes was autophosphorylated for 5 min at 20 μM [γ-^{32}P]ATP, in the absence (open symbols) or presence of 1 μM insulin (closed symbols). Control reactions were done in the absence of polypeptide substrates. Reactions were done at the indicated final concentrations of RCAM-lysozyme (○, ●) or angiotensin I (△, ▲). Maximum autophosphorylation is the amount of ^{32}P-labeled β-subunit recovered after the reaction at 1 μM insulin; at a specific activity of 185 cpm/fmol, basal-state ^{32}P-β-subunit = 3070 ± 400 cpm, and insulin-stimulated ^{32}P-β-subunit = 23 600 ± 750 cpm.

shown]. This should render the nitrotyrosine too weak a nucleophile to participate in phosphoryl transfer. Support for this latter hypothesis came from reduction of the nitro to the amino group, which restored the pK' to ≈10 (Sokolovsky et al., 1967) and also restored phosphorylation of the peptide aminotyrosyl-V8-3 (data not shown).

Different Effects of Two Polypeptide Substrates on Autophosphorylation. According to the proposal of Bertics and Gill (1985), we tentatively concluded that inhibition of autophosphorylation by these substrates was due to competitive inhibition; i.e., occupancy of the phosphoryl transfer site by the dissociable peptide substrates in place of the nondissociable segments of the receptor that are *auto*phosphorylated. Therefore, angiotensin and RCAM-lysozyme as substrates should each inhibit receptor autophosphorylation. We also presumed that RCAM-lysozyme with a $K_{M,app} \approx 10$ μM (Kohanski & Lane, 1986) would be a higher affinity inhibitor of receptor autophosphorylation than angiotensin with a $K_{M,app} \approx 1$ mM, also measured at 20 μM [γ-^{32}P]ATP. Autophosphorylation of native insulin receptor purified from 3T3-L1 mouse adipocytes was inhibited by the substrate angiotensin; 50% inhibition was observed at 0.9 and 1.3 mM angiotensin, without or with insulin, respectively (Figure 3).

With RCAM-lysozyme present during the reaction, receptor autophosphorylation displayed two surprising patterns (Figure 3). First, the inhibition in the presence of 1 μM insulin did not approach completion although an $IC_{50} \approx 0.6$ μM could be stated since that concentration of RCAM-lysozyme gave 50% inhibition of maximum autophosphorylation. However, there was no additional inhibition between 1 and 100 μM RCAM-lysozyme. Second, autophosphorylation was *stimulated* in the absence of insulin. It must be stressed that in this and the following experiments there was no stimulation by peptide substrates when the receptor was saturated with insulin.

While a relatively simple peptide substrate such as angiotensin may act solely as a competitive inhibitor of autophosphorylation, the effects of more complex peptide substrates such as RCAM-lysozyme indicate that multiple levels of interaction with the insulin receptor are possible. There may be a link between stimulation and the maximum extent of inhibition achieved by RCAM-lysozyme, compared to that by angiotensin. However, to better understand this stimulation we prepared and tested derivatives of RCAM-lysozyme.

Modifications of the Substrate That Altered Inhibition and Stimulation. Cleavage of RCAM-lysozyme with V8 proteinase yielded two fragments containing tyrosyl groups (Figure 1). With either V8-3 or V8-2 we observed inhibition of receptor autophosphorylation in the presence of insulin (Figure 4, upper panel). As described above, V8-2 (residues 8–35) was not a substrate for this protein kinase. In addition, V8-2 did not stimulate autophosphorylation in the absence of insulin, although inhibition was observed; $IC_{50} \approx 30$ and 50 μM V8-2 in the presence and absence of insulin, respectively (Figure 4, upper panel). The substrate polypeptide V8-3 inhibited autophosphorylation in the presence of insulin (Figure 4, upper panel); $IC_{50} \approx 1.5$ μM V8-3. In the absence of insulin, a 2-fold stimulation of receptor autophosphorylation was observed. This magnitude of stimulation of the basal reaction was the same as observed with RCAM-lysozyme, although the stimulation was observed over a narrower concentration range (compare Figure 4 with Figure 3).

Cleavage of RCAM-lysozyme with CNBr (Figure 1) yielded two fragments that altered insulin receptor autophosphorylation. The peptide CB-3 lacks tyrosine and was not a substrate. However, CB-3 inhibited both the basal and insulin-stimulated reactions ($IC_{50} \approx 50$ μM, with or without insulin) but did not itself stimulate basal autophosphorylation (Figure 4, lower panel). The fragment CB-2 was a substrate, an inhibitor of autophosphorylation in the presence of insulin ($IC_{50} \approx 0.6$ μM), and a stimulator of the basal autophosphorylation reaction, with a maximal effect between 0.5

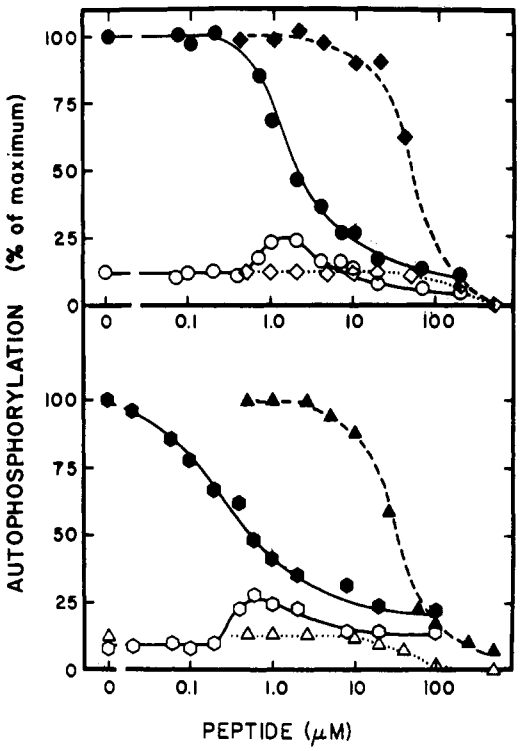

FIGURE 4: Stimulation and inhibition of autophosphorylation using primary cleavage fragments of RCAM-lysozyme. (Upper panel) Native insulin receptor from *mouse* 3T3-L1 adipocytes was autophosphorylated for 5 min at 10 μM [γ-^{32}P]ATP, in the absence (open symbols) or presence (closed symbols) of 1 μM insulin at the indicated concentrations of V8-3 (○, ●) or V8-2 (◇, ◆). Control maximum versus basal autophosphorylation (i.e., plus versus minus insulin) was 7.8 ± 0.6. Basal autophosphorylation yielded 962 ± 52 cpm, from 10 μM [γ-^{32}P]ATP at 98 cpm/fmol. (Lower panel) Native *human* insulin receptor was autophosphorylated as in panel A, in the presence (closed symbols) or absence (open symbols) of 1 μM insulin, at the indicated concentrations of CB-2 (○, ●) or CB-3 (△, ▲). Reactions with CB-2 were done at a specific activity of ≈310 cpm/fmol of [γ-^{32}P]ATP, yielding basal autophosphorylation of 1060 ± 97 cpm and stimulated autophosphorylation of 12 400 ± 240 cpm. Reactions with CB-3 were under similar conditions, but where the average fold stimulation by insulin was (8.0 ± 0.5)-fold.

and 2 μM (Figure 4, lower panel). Although the data are shown with purified receptor from two different mammalian origins (Figure 4, upper versus lower panel), the parameters for inhibition and for stimulation were nearly the same whether the murine or human receptor was used. Thus, it was found that the IC_{50} of CB-2 was ≈2-fold lower than the IC_{50} of V8-3, and stimulation was observed first at a slightly lower concentration of CB-2 than of V8-3, although always near 1 μM peptide.

The results presented thus far for V8-3 and CB-2 led us to propose that the stimulatory region of RCAM-lysozyme would be in the amino acid sequence overlapping V8-3 and CB-2; i.e., residues 36–105 which constitute the secondary cleavage product VC-1 (Figure 1). When tested with the human insulin receptor, VC-1 displayed neither inhibition nor stimulation of receptor autophosphorylation (Figure 5, upper panel). However, when tested with purified insulin receptor from mouse 3T3-L1 cells, inhibition of the reaction by VC-1 was observed in the presence of insulin with an $IC_{50} \approx 20$ μM, but neither inhibition nor stimulation was detected for the basal reaction (Figure 5, inset). Because VC-1 was derived from V8-3 by CNBr cleavage, we attempted to reconstitute stimulation by a combination of VC-1 with CB-3. This pair contains in two fragments the primary structure of V8-3. Covering concentration ranges of 1–25 μM for both peptides,

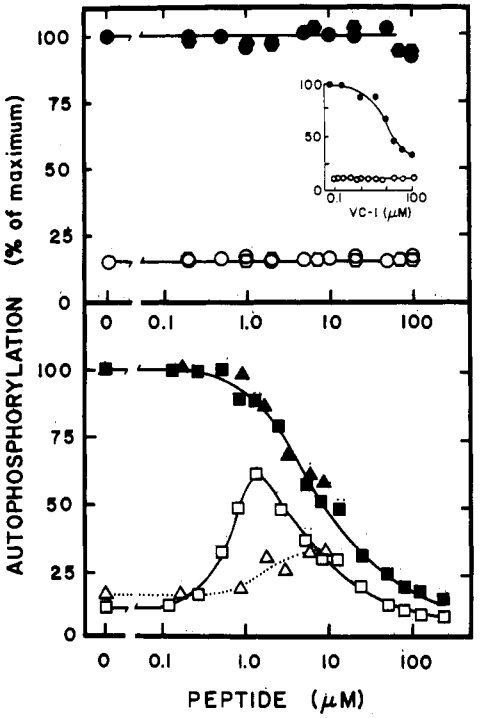

FIGURE 5: Autophosphorylation in the presence of derivatives of V8-3. (Upper panel) Native *human* insulin receptor was autophosphorylated for 5 min at 10 μM [γ-^{32}P]ATP and 5 mM $Mn(CH_3CO_2)_2$, in the absence (open symbols) or presence (closed symbols) of 1 μM insulin. Reactions were done at the indicated concentrations of maleyl-V8-3 (○, ●) or VC-1 (○, ●). The autophosphorylation reactions with VC-1 were done with a specific activity of 195 cpm/fmol of [γ-^{32}P]ATP, yielding basal and stimulated ^{32}P-β-subunit of 1400 ± 100 and 12 230 ± 770 cpm, respectively. Reactions with maleyl-V8-3 were done with a specific activity of 85 cpm/fmol of [γ-^{32}P]ATP, yielding 2190 ± 100 and 16 900 ± 690 cpm in the basal and insulin-stimulated states, respectively. (Insert) Native mouse 3T3-L1 insulin receptor was autophosphorylated as above, at the indicated concentrations of VC-1, in the absence (○) or presence (●) of 1 μM insulin. (Lower panel) Autophosphorylation of *mouse* 3T3-L1 receptor, as above, was done at the indicated concentrations of nitrotyrosyl-V8-3 (□, ■) or aminotyrosyl-V8-3 (△, ▲) in the absence (open symbols) or presence (closed symbols) of 1 μM insulin. One hundred percent of maximum was determined in the absence of polypeptide substrate at 1 μM insulin. Control maximum versus basal autophosphorylation (i.e., plus versus minus insulin) was 7.8 ± 0.6. Basal autophosphorylation yielded 962 ± 52 cpm from 10 μM [γ-^{32}P]ATP at 98 cpm/fmol.

in all combinations, did not stimulate the basal reaction (data not shown). Therefore to achieve stimulation, V8-3 must be intact.

Following this conclusion, we tested two other chemically modified forms of V8-3. We found that maleyl-V8-3 did not stimulate the basal autophosphorylation reaction and was also a weak inhibitor of the insulin-stimulated reaction (Figure 5, upper panel). From this, we conclude that not only must V8-3 be intact but that positively charged groups are important for both the inhibition and stimulation observed with V8-3. Whereas the site of substrate phosphorylation was at Tyr-53, the newly modified amino groups (besides the α-amino group of residue 36) were the lysyl ε-amino groups at positions 96, 97, and 116 which are ≥43 residues removed from the phosphorylatable tyrosine.

We then tested modification of the tyrosine in V8-3 that is phosphorylated by the insulin receptor. Nitrotyrosyl-V8-3 was a weaker inhibitor of receptor autophosphorylation,[3] re-

[3] With the cloned human insulin receptor, ≈2.5 μM nitrotyrosyl-V8-3 gave 50% inhibition (at 1 μM insulin), and ≈1 μM nitrotyrosyl-V8-3 yielded maximal 3–4-fold stimulation in the absence of insulin.

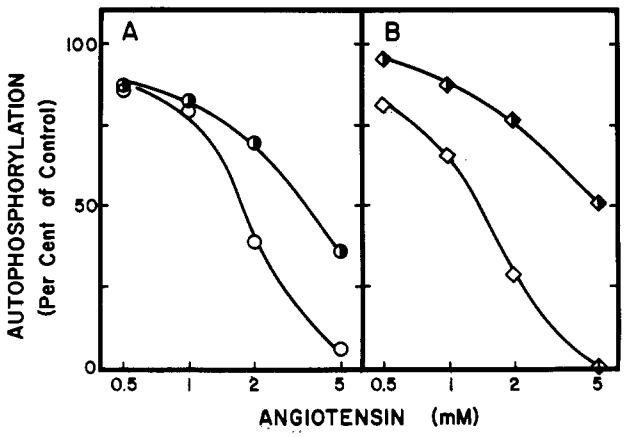

FIGURE 6: Changes in the inhibition by a simple peptide substrate. Purified human insulin receptor was autophosphorylated for 5 min at 10 μM [γ-^{32}P]ATP (specific activity $\approx$ 64 cpm/fmol) and 5 mM $Mn(CH_3CO_2)_2$ at the indicated concentrations of angiotensin, in the absence (open symbols) or presence (half-filled symbols) of nitrotyrosyl-V8-3. Results are expressed relative to each control reaction, performed without angiotensin. (Panel A) Reactions in the absence of insulin, with (◑) or without (○) 2 μM nitrotyrosyl-V8-3. The control reactions yielded the following autophosphorylation: basal (without nitrotyrosyl-V8-3), 560 ± 45 cpm; with nitrotyrosyl-V8-3, 1650 ± 125 cpm. (Panel B) Reactions at 1 μM insulin, with (half-filled diamond) or without (◇) 5 μM nitrotyrosyl-V8-3. The control reactions yielded the following autophosphorylation: without nitrotyrosyl-V8-3, 6020 ± 430 cpm; with nitrotyrosyl-V8-3, 1820 ± 125 cpm.

quiring $\approx$4.8 μM nitrotyrosyl-V8-3 to achieve 50% inhibition (Figure 5, lower panel). The net stimulation of basal autophosphorylation by nitrotyrosyl-V8-3 was greater than that with V8-3, although maximum stimulation was observed at about the same concentration of peptide (compare Figures 4 and 5). We noted above that nitrotyrosyl-V8-3 was not phosphorylated by this receptor and that reduction of the nitro group, yielding aminotyrosyl-V8-3, restored the phosphoacceptor character of this peptide. Aminotyrosyl-V8-3 was at best sparingly soluble and therefore was not useful for extensive characterization. However, aminotyrosyl-V8-3 yielded a 2-fold rather than a 5-fold stimulation of receptor autophosphorylation, but at slightly higher peptide concentrations and without significant changes in its inhibitory characteristics (Figure 5, lower panel). Comparison of results from both human and murine receptor showed that stimulation by nitrotyrosyl-V8-3 was consistently $\approx$2-fold greater than stimulation by unmodified V8-3. Thus, residues near the amino and carboxy termini of V8-3 have significant roles in the observed stimulation of autophosphorylation.

Combined Effects of a Stimulatory Peptide and a Substrate Inhibitor on Autophosphorylation. We tested the ability of nitrotyrosyl-V8-3 to stimulate receptor autophosphorylation under conditions where the reaction was inhibited by a peptide substrate. The concentrations of nitrotyrosyl-V8-3 employed in this experiment were selected to give optimal stimulation of the basal autophosphoryltion reaction and to give pronounced inhibition of the insulin-stimulated reaction (see Figure 5). The peptide angiotensin was used because it yielded inhibition but not stimulation of autophosphorylation (see Figure 3). Fifty percent inhibition of autophosphorylation was observed at 1.7 and 1.4 mM angiotensin, for control reactions in the absence or presence of 1 μM insulin, respectively. However, higher concentrations of angiotensin were required to achieve 50% inhibition when nitrotyrosyl-V8-3 was present (Figure 6). For the sake of comparison, these data are expressed as percent of control autophosphorylation. The control reaction at 2 μM nitrotyrosyl-V8-3 and without angiotensin

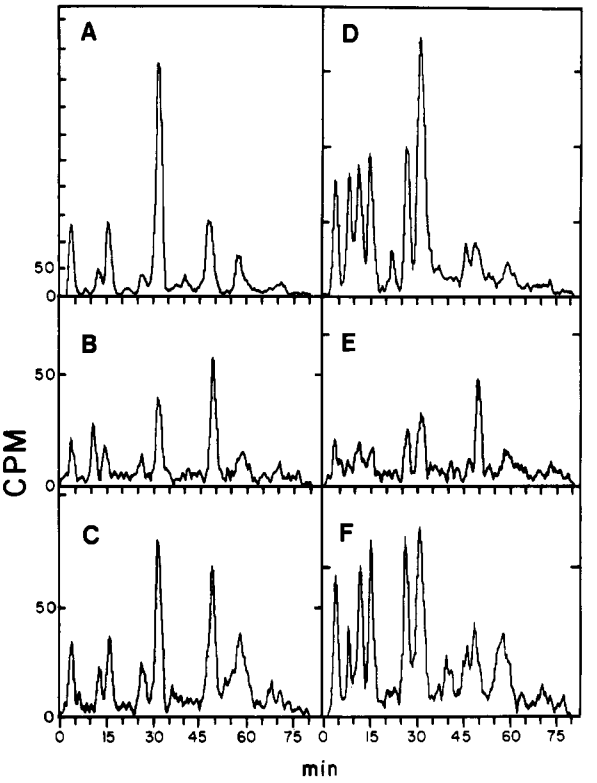

FIGURE 7: ^{32}P-Labeled phosphopeptides of native insulin receptor at 2 and 50 μM [γ-^{32}P]ATP. Native *human* insulin receptor was autophosphorylated for 5 min at 5 mM $Mn(CH_3CO_2)_2$, using 2 μM [γ-^{32}P]ATP (panels A–C), or at 50 μM [γ-^{32}P]ATP (panels D–F). Reactions: stimulated with 1 μM insulin (A and D); basal (B and E); stimulated with 2 μM nitrotyrosyl-V8-3 (C and F). Preparation of the tryptic phosphopeptides and the chromatography conditions are described under Experimental Procedures. Each hash mark indicates 50 cpm.

was 2.9-fold stimulated above basal. The control reaction at 5 μM nitrotyrosyl-V8-3 but without angiotensin was inhibited 70% relative to the control insulin-stimulated reaction.

Phosphopeptide Maps of the Native Human Insulin Receptor. Following autophosphorylation at 2 versus 50 μM [γ-^{32}P]ATP, certain differences were found in the relative distribution of ^{32}P-labeled phosphopeptides (Figure 7). It was hard to distinguish differences in the maps representing basal (unstimulated) autophosphorylation performed at 2 versus 50 μM [γ-^{32}P]ATP (panel B versus panel E of Figure 7). However, it was easy to find differences in maps representing insulin-stimulated autophosphorylation at 2 versus 50 μM [γ-^{32}P]ATP (panel A versus panel D of Figure 7). Nevertheless, in a comparison of insulin-stimulated to basal autophosphorylation, there was a definite signature of the insulin-stimulated autophosphorylation state at each concentration (panel A versus panel B and panel D versus panel E of Figure 7).

The phosphopeptide maps resulting from nitrotyrosyl-V8-3 stimulation were similar to those from insulin stimulation, at the corresponding concentrations of ATP (panel C versus panel A and panel F versus panel D of Figure 7). Although the *net* stimulation of autophosphorylation was readily observed at all tested concentrations of [γ-^{32}P]ATP, the "sitewise" effects were better demonstrated at the higher concentration. Thus, under the 5-min reaction conditions used here, maximum stimulation by V8-3 was not significantly dependent upon the ATP concentration: At 2 μM [γ-^{32}P]ATP the stimulation was (2.0 ± 0.4)-fold above basal and (1.6 ± 0.3)-fold at 50 μM [γ-^{32}P]ATP. Both were similar in magnitude to the (2.0 ±

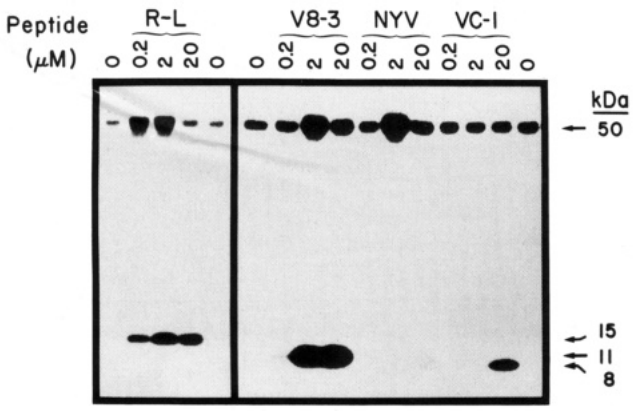

FIGURE 8: Autophosphorylation of the cloned cytoplasmic domain of the protein kinase (R-BIRK). Autophosphorylation of R-BIRK was done for 5 min at 10 μM [γ-^{32}P]ATP. The products were separated by SDS–PAGE, as described under Experimental Procedures. The autoradiograms show two separate experiments: (left) ≈26 ng of R-BIRK (≈18 nM), 70-min exposure; (right) ≈21 ng of R-BIRK (≈15 nM), 3-h exposure. The ^{32}P-labeled products were identified by their apparent molecular masses, given in kDa on the right of the figure: R-BIRK, 50 kDa; R-L (RCAM-lysozyme), 15 kDa; V8-3, 11 kDa; VC-1, 8 kDa. NYV (nitrotyrosyl-V8-3), at 11 kDa, was not phosphorylated. The polypeptides employed, and their concentrations (μM), are indicated above each lane. Control reactions ("0" μM) were without added polypeptides.

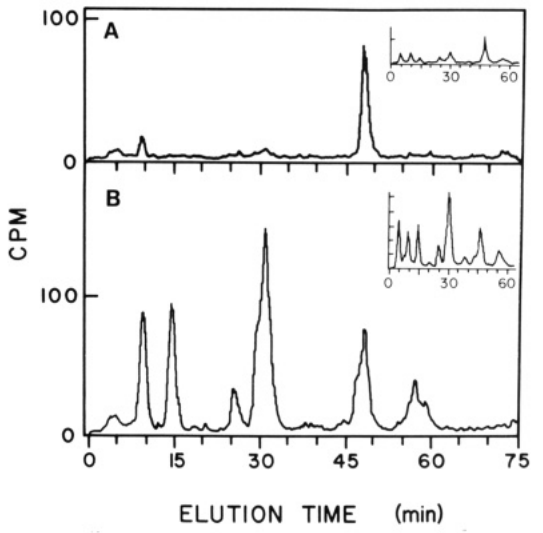

FIGURE 9: ^{32}P-Labeled phosphopeptides of the cloned cytoplasmic kinase domain. Autophosphorylation of 79 ng of R-BIRK (≈55 nM) was done for 10 min at 10 μM [γ-^{32}P]ATP (≈240 cpm/fmol). The conditions for digestion with trypsin and separation of the products by HPLC are given under Experimental Procedures. (Panel A) No additions (basal state); (panel B) at 2 μM nitrotyrosyl-V8-3. Stimulated autophosphorylation was ≈4.5-fold above basal. The insets show native human insulin receptor autophosphorylated for 10 min at 10 μM [γ-^{32}P]ATP in the absence or presence of 1 μM insulin (panels A and B, respectively).

0.4)-fold stimulation observed at 10 μM [γ-^{32}P]ATP (Figure 4, upper panel). Maximum stimulation was consistently observed between 1 and 2 μM V8-3. The same finding was true for nitrotyrosyl-V8-3; the latter was used for phosphopeptide mapping because it gave a higher fold stimulation than unmodified V8-3.

Stimulation of the Cloned Cytoplasmic Domain of the Human Insulin Receptor. The following experiments demonstrated that stimulatory peptides interact with the cytoplasmic domain of the receptor. For this we used R-BIRK, generated and characterized by Rosen and co-workers (Villalba et al., 1989). R-BIRK lacks the insulin-binding α-subunit and the extracellular and transmembrane domains of the β-subunit. The observed stimulation of autophosphorylation by RCAM-lysozyme, V8-3, and nitrotyrosyl-V8-3 [(6.5 ± 1.1)-fold above control] showed that these polypeptides interact with the protein kinase domain as stimulators (Figure 8). As with native human insulin receptor, increasing concentrations of peptides above the concentration giving greatest stimulation resulted in decreased levels of autophosphorylation, relative to the maximally stimulated state (Figure 8). Most importantly, these results eliminate the possibility that stimulation of the native receptor resulted from interaction with the insulin-binding α-subunit.

In addition, it can be seen in these autoradiograms (Figure 8) that nitrotyrosyl-V8-3 was not phosphorylated and that VC-1 was itself a substrate but did not stimulate or inhibit R-BIRK autophosphorylation. The characteristics of interaction of substrate peptides with the intact receptor were thus also found in their interaction with R-BIRK. That is, V8-3 is the minimum polypeptide that yields stimulation, and R-BIRK has those features essential to show stimulation. In contrast, the nonsubstrate peptides V8-2 and CB-3 had no detectable effect on R-BIRK autophosphorylation at concentrations up to 250 μM (not shown), although they did inhibit autophosphorylation of the native insulin receptor, in the absence or presence of insulin (Figure 4). It is therefore probable that their inhibition of native receptor autophosphorylation required binding to a region of the kinase removed by the cloning protocol; i.e., the transmembrane and/or extracellular domains of the β-subunit and possibly the α-subunit.

Phosphopeptide Maps from the Cloned Cytoplasmic Domain of the Human Insulin Receptor. The phosphopeptide map generated after autophosphorylation of R-BIRK at 5 mM $Mn(CH_3CO_2)_2$ and 10 μM [γ-^{32}P]ATP alone was similar to that of native insulin receptor in its basal state (Figure 9A). The phosphopeptide map generated after nitrotyrosyl-V8-3 stimulation of R-BIRK was strikingly similar to that of native insulin receptor stimulated by insulin under the same reaction conditions (Figure 9B). These findings are consistent with peptide stimulation being effective on the enzyme in its basal state. This stimulation is comparable to that induced in the native receptor by insulin. Therefore, since peptide and insulin produced similar "sitewise" autophosphorylation in R-BIRK and the native receptor, respectively, these peptides allow insulin-like stimulation of R-BIRK even though the cytoplasmic kinase domain is intrinsically incapable of binding insulin.

Discussion

The polypeptide substrate RCAM-lysozyme interacts with the insulin receptor in a complex manner. In this study we showed that basal autophosphorylation was stimulated by RCAM-lysozyme and selected derivatives. These same peptides were inhibitors of autophosphorylation in the presence of insulin. We shall consider whether or not these effects are due exclusively to the substrate character of the peptides, a possible mechanism for stimulation, and our evidence that supports binding of stimulatory peptides to the cytoplasmic domain of the receptor.

The Substrate's Phosphorylation Site. We established that Tyr-53 and not Tyr-20 or Tyr-23 is the site of phosphorylation on RCAM-lysozyme. Clearly, a single tyrosine does not define all the substrate binding properties of these polypeptides but only their competence to accept phosphate from ATP [cf. Walker et al. (1986)]. The charged amino acids preceding both Tyr-20 and Tyr-53 each conform to the distribution important for substrate recognition by protein (tyrosine) ki-

nases (Hunter & Cooper, 1985). Also, we note from the crystal structure of native lysozyme that Tyr-53 is in a β-strand, but it may be that tyrosines in β-turns are preferred by protein (tyrosine) kinases (Tinker et al., 1988), where Tyr-20 and Tyr-23 are found. Even though two tyrosines are likely to be recognized as substrates, Tyr-53 is the only tyrosine residues of RCAM-lysozyme phosphorylated by the insulin receptor under the reported reaction conditions. This fact might aid in the future design of peptide substrates.

Inhibition of Autophosphorylation. Not all peptides containing Tyr-53 were equally effective as inhibitors of receptor autophosphorylation. Compared with V8-3, nitrotyrosyl-V8-3 and aminotyrosyl-V8-3 were weaker inhibitors. We concluded that steric constraints due to ortho substitution on Tyr-53 weakened the inhibition (see Results). Thus, some modifications at the phosphoryl acceptor site of the substrate appear to be correlated with changes in the inhibition of autophosphorylation. This would be consistent with competitive inhibition of autophosphorylation by a diffusible peptide substrate.

However, alterations in V8-3 made 40–50 amino acids distal to the phosphorylation site had dramatic effects on inhibition, specifically VC-1 and maleyl-V8-3. In both peptides the phosphorylation site was intact, and both yielded apparent Michaelis constants of 25 μM, measured at 20 μM [γ-^{32}P]-ATP. Compared with a Michaelis constant of 10 μM for RCAM-lysozyme, V8-3 and CB-2 (measured at 20 μM [γ-^{32}P]ATP), modifications giving maleyl-V8-3 and VC-1, had a small effect on the substrate character of these peptides but a larger effect on their abilities to inhibit autophosphorylation. Therefore, high-affinity inhibition by RCAM-lysozyme was most likely due to the substrate character *and* other regions of RCAM-lysozyme structure different from the phosphorylation site at Tyr-53.

Stimulation of Autophosphorylation. Stimulation of basal receptor autophosphorylation was observed with the peptides RCAM-lysozymes, CB-2, V8-3, nitrotyrosyl-V8-3, and aminotyrosyl-V8-3. As the overlapping peptide common to the stimulators V8-3 and CB-2, we had predicted that VC-1 would also stimulate autophosphorylation, yet it did not. Furthermore, maleyl-V8-3 did not stimulate autophosphorylation. Each of these peptides contained Tyr-53 and, therefore, the substrate region of RCAM-lysozyme. Thus, the substrate region per se was insufficient to induce stimulation. Since the segments removed from V8-3 and CB-2 were not substrates (CB-3 and V8-2, respectively), they were tested but shown not to stimulate autophosphorylation; indeed, they were inhibitors (Figure 4). Finally, mixtures of CB-3 with VC-1 (the structural components of V8-3) did not stimulate autophosphorylation. Therefore, it appears that two contiguous structural regions of RCAM-lysozyme must be present on a *single* polypeptide to stimulate insulin receptor autophosphorylation.

There is also a requirement for selected positively charged residues to achieve stimulation. Maleyl-V8-3 contained four modified primary amino groups—none close to Tyr-53—and was incapable of stimulating autophosphorylation. Because VC-1 contained the unmodified α-amino group and lysyl residues at positions 96 and 97 (of RCAM-lysozyme) yet lacked the lysine at residue 116 *and* was not a stimulator, we conclude that Lys-116 plays a role in the stimulation of autophosphorylation.

We propose that peptide stimulation of autophosphorylation is best correlated with the "anti-inhibition" described in Figure 6. The stimulatory peptide, nitrotyrosyl-V8-3, may have caused increased occupancy of the catalytic center with the receptor's autophosphorylation sites which would have excluded the competitive inhibitor angiotensin. This yielded the increased autophosphorylation: in effect, competitive inhibition against the substrate. The corollary for this is that peptide stimulation results from increasing the entry of autophosphorylation sequences into the catalytic center, even when there are no diffusible substrates/competitive inhibitors present.[4] Such an experimental situation, with a substrate/inhibitor present, was the only one in which peptide stimulation was observed in the presence of insulin. We could suggest, post hoc, that insulin also has the effect of increasing the autophosphorylation sites' effective concentration in the catalytic center, which could certainly explain increased rates of autophosphorylation. However, insulin did not change the titration of inhibition by angiotensin, compared to that with the absence of insulin. Again, nitrotyrosyl-V8-3, even in the presence of insulin, altered the inhibition by angiotensin. Because of these findings and despite the fact that stimulation by peptides was not found to be additive with stimulation by insulin, we conclude reluctantly that the mechanism for peptide stimulation differs from the mechanism for insulin stimulation.

Phosphopeptide Maps after Insulin or Peptide Stimulation. We have shown that both insulin and the polypeptides produced comparable sitewise effects in the autophosphorylation of native insulin receptor. Even though the hierarchy of sites autophosphorylated is dependent upon ATP concentration, stimulation by insulin or by peptides yielded similar patterns of autophosphorylation, as demonstrated in Figure 6. It was previously shown that stimulation by poly(L-lysine) produced similar phosphopeptide maps after autophosphorylation as did insulin (Kohanski, 1989). In that case stimulation by poly(L-lysine) was found to occur in the presence of insulin. Poly(L-lysine) also increased the near-equilibrium level of autophosphorylation. In contrast, the presence of 2 μM nitrotyrosyl-V8-3 did not alter the near-equilibrium level of autophosphorylation in the absence or presence of insulin, measured at 10–100 μM ATP (data not shown). Therefore, despite the requirement for selected positive charges on V8-3, stimulation by these peptides must also differ from stimulation by polycations such as poly(L-lysine). It is important to note that comparable phosphopeptide maps are not by themselves sufficient criteria to infer that truly insulin mimetic activation has taken place.

Analysis of the ^{32}P-labeled phosphopeptide maps generated from R-BIRK suggests three important conclusions: First, the soluble cytoplasmic domain of the insulin receptor is autophosphorylated as a basal-state enzyme, i.e., in an *un*stimulated state that is comparable to the native receptor in its unstimulated state. This conclusion was reached by comparison of phosphopeptide maps generated after autophosphorylation of R-BIRK and of native insulin receptor without insulin (Figure 9A). Equally important to this conclusion was the observation that peptide-stimulated R-BIRK and insulin-stimulated native receptor yielded similar maps (Figure 9B). Since we do not know the level or sites of autophosphorylation in this preparation of R-BIRK, it is possible that a basal-state phosphopeptide map might have been found

[4] Increasing the peptide concentration increases both the inhibitor and the anti-inhibitor concentrations. Thus, the property of being an anti-inhibitor may explain the inability of RCAM-lysozyme and its derivatives to yield nearly complete inhibition of autophosphorylation even at concentrations 50-fold above the IC_{50}, whereas a simple peptide such as angiotensin is capable of nearly complete inhibition at concentrations of $\approx$10-fold above the IC_{50}.

if certain sites were already autophosphorylated and thus unavailable for the in vitro reaction. Therefore, obtaining the "insulin-stimulated" phosphopeptide map with peptide-stimulated R-BIRK indicates that those sites were indeed available, but were less reactive in the absence of peptide.

Second, peptide stimulation of R-BIRK yields an autophosphorylation state that mimics the insulin-stimulated state of the native $\alpha_2\beta_2$ receptor (Figure 9B). The most important conclusion is that all structural information required for the kinase to be stimulated by these peptides is contained in the cytoplasmic domain of the receptor. We cannot yet determine if R-BIRK always autophosphorylates as a monomeric enzyme [see Villalba et al. (1989)]. If true, this would indicate separate stimulatory and inhibitory binding sites on the enzyme for the peptides described here. If on the other hand these peptides drive R-BIRK monomers to interact [interaction between monomers was proposed by Cobb et al. (1989)], there may then be cooperative interactions between kinase domains and/or the phosphorylation of one domain by another. Future work will address these issues directly.

Third, peptide stimulation of R-BIRK autophosphorylation is a practical means to study activation of this domain of the insulin receptor. The enzyme can be separated readily from the activating peptides by ion exchange or gel permeation chromatography.

The stimulatory peptides described in this paper will also be useful tools for the study of mutant insulin receptors, especially those with defects in insulin stimulation of autophosphorylation.[5] However, cloned human insulin receptor present in the wheat germ agglutinin–Sepharose eluate is stimulated with nitrotyrosyl-V8-3 by only 30% compared to the observed stimulation of 300–450% with highly purified receptor. Careful attention must be given to the state of receptor purity before these peptides can be employed.

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