Substrate Phosphorylation Catalyzed by the Insulin Receptor Tyrosine Kinase

KINETIC CORRELATION TO AUTOPHOSPHORYLATION OF SPECIFIC SITES IN THE β SUBUNIT

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The kinetics of insulin-stimulated autophosphorylation of specific tyrosines in the β subunit of the mouse insulin receptor and activation of receptor kinase-catalyzed phosphorylation of a model substrate were compared. The deduced amino acid sequence of the mouse proreceptor was determined to locate tyrosine-containing tryptic peptides. Receptor was first incubated with unlabeled ATP to occupy nonrelevant autophosphorylation sites, after which [32P]autophosphorylation at relevant sites and attendant activation of substrate phosphorylation were initiated with [γ-32P]ATP and insulin. Activation of substrate phosphorylation underwent an initial lag of 10–20 s during which there was substantial 32P-autophosphorylation of tryptic phosphopeptides p2 and p3, but not p1. Following the lag, incorporation of 32P into p1 and the activation of substrate phosphorylation increased abruptly and exhibited identical kinetics. The addition of substrate to the receptor prior to ATP inhibits insulin-stimulated autophosphorylation, and consequently substrate phosphorylation. Insulin-stimulated autophosphorylation of the receptor in the presence of substrate inhibited primarily the incorporation of 32P into p1 and drastically inhibited substrate phosphorylation. From Edman radiolabeling of 32P-labeled p1, p2, and p3 and the amino acid sequence of the mouse receptor, the location of each phosphopeptide within the β subunit was determined. Further characterization of these phosphopeptides revealed that p1 and p2 represent the triply and doubly phosphorylated forms, respectively, of the region within the tyrosine kinase domain containing tyrosines 1148, 1152, and 1153. The doubly phosphorylated forms contain phosphotyrosines either at positions 1148 and 1152/1153 or positions 1152 and 1153. These results indicate that insulin stimulates sequential autophosphorylation of tyrosines 1148, 1152, and 1153, and that the transition from the doubly to the triply phosphorylated forms is primarily responsible for the activation of substrate phosphorylation.

Upon binding to its specific cell-surface receptor, insulin initiates a pleiotropic cellular response, notably the activation of energy storage processes including glucose uptake, glycogen synthesis, and lipogenesis (1). The insulin receptor is a transmembrane allosteric enzyme composed of two types of subunits, i.e., α and β subunits, that are stabilized in a βα-α-β tetrameric structure by intersubunit disulfide bonds (2, 3). The α subunit which is entirely extracellular contains the insulin-binding site, while the β subunit which spans the plasma membrane houses a cytoplasmic tyrosine kinase catalytic domain (4, 5). Studies with purified insulin receptor preparations (6–8) and with intact cells (9, 10) have shown that the interaction of insulin with its binding site in the α subunit stimulates autophosphorylation of multiple tyrosyl groups within the intracellular kinase domain.

The mechanism by which the insulin-induced allosteric signal is transmitted across the plasma membrane is unknown. Nevertheless, there is substantial evidence (11–14) that insulin-stimulated autophosphorylation of the β subunit causes activation of kinase-catalyzed phosphorylation of model protein substrates and presumably cellular protein substrates. Thus, Rosen et al. (11) first demonstrated that the lag in insulin-activated substrate phosphorylation (initiated with ATP) is eliminated by prior incubation of the receptor with insulin and ATP. Since the receptor underwent autophosphorylation (on tyrosine) during preliminary incubation, it appeared that insulin-stimulated autophosphorylation might be an essential step in the activation process. This interpretation was substantiated by Yu and Czech (12) who showed that removal of the phosphoryl groups (added during autophosphorylation) with alkaline phosphatase reversed the activation of substrate phosphorylation.

Compelling evidence that insulin-stimulated autophosphorylation induces activation of substrate phosphorylation has been obtained using protein substrates as inhibitors of insulin-stimulated autophosphorylation (15–18). For example, it was shown (15, 16) that RCAM-lysozyme, which is an excellent model substrate of the insulin receptor tyrosine kinase (Kin~ 10 μM), is also a potent inhibitor of insulin-stimulated autophosphorylation (Kin~ 1 μM). Thus, RCAM-lysozyme, added prior to ATP and insulin, totally blocks insulin-stimulated autophosphorylation and as a consequence, blocks insulin-stimulated substrate phosphorylation (15, 16). By taking advantage of these properties of RCAM-lysozyme, the dependence of substrate phosphorylation capacity upon frac-
tional autophosphorylation (stimulated by insulin) of the receptor was demonstrated. It was also determined that maximal insulin-stimulated autophosphorylation and substrate phosphorylation capacity were achieved before all autophosphorylation sites had been occupied. This finding revealed that not all sites of autophosphorylation are involved in the activation process and prompted efforts to identify the responsible sites.

Knowledge of the complete amino acid sequence of the human insulin receptor (4, 5) greatly facilitated identification of tyrosyl autophosphorylation sites within the β subunit. Thus, it became possible to locate candidate autophosphorylation sites based on consensus amino acid sequences at tyrosyl residues phosphorylated by other tyrosine-specific kinases. Moreover, by analysis of [32P]phosphopeptides generated by proteolytic cleavage of the 32P-labeled receptor β subunit at sites predicted by the primary sequence, it has been determined that at least 6 tyrosyl residues undergo phosphorylation during insulin-stimulated autophosphorylation (19). Most of these tyrosines are located in two segments of the β subunit. One segment is located within the putative catalytic tyrosine kinase domain and contains tyrosines 1146, 1150, and 1151 which are phosphorylated by the receptor tyrosine kinase (21). The other segment contains tyrosines 1316 and 1322 near the COOH-terminal end of the β subunit (19). A number of investigations (see “Discussion” and Refs. 20–23) have implicated autophosphorylation at tyrosines 1146, 1150, and 1151 in the activation of protein substrate phosphorylation catalyzed by the receptor.

In the present study, we have identified the earliest autophosphorylation events, i.e. between 0 and 60 s, following the phosphorylation at specific sites in the receptor subunits. Partial phosphorylation of this domain (i.e. tyrosines 1148, 1152, and 1151 in the human insulin receptor) is followed by 1345 residues, compared with 1343 residues in the mouse receptor, which gives rise to the α and β subunits of the mature receptor (4). The two additional amino acids (threonine and proline) in the mouse receptor are inserted in the α subunit following a glutamine residue at position 546, thereby shifting the numbering beyond this point in the mouse receptor by +2 amino acid residues with respect to the human receptor. The overall amino acid sequence identity between the two receptors is 95%, with the α and β subunits exhibiting 97% and 94% identity, respectively. The positions of the 36 cysteine residues in the α subunit are totally conserved in both receptors, while 9 of 10 cysteines in the β subunit of the human receptor are conserved in the mouse receptor with the exception of cysteine 971, which is substituted by a serine residue. The number of relative positions of potential N-linked glycosylation sites are the same in both receptors, and the sequence of the COOH terminus of the α subunit, which contains the putative proteolytic cleavage site and gives rise to the α and β subunits, is totally conserved. The transmembrane sequences of the β subunit in both receptors are identical, and the intracellular domain, which contains the tyrosine kinase catalytic site, is highly conserved exhibiting 95% amino acid sequence identity. The intracellular segment of the β subunit, which begins at arginine 943 (941 in the human proreceptor) contains a consensus ATP-binding site (Gly-X-Gly-X-X-Gly; residues 993–998), 50-amino acid residues from the transmembrane domain. The β subunit also contains a lysine residue at position 1020 that is equivalent to lysine 1018 in the human proreceptor which has been shown to be essential for tyrosine kinase activity (31). The only region of significant sequence divergence between the mouse and the human receptors is at the NH2 terminus of the β subunit encompassing residues 728–735 (presumably an extracellular region), where the amino acid sequence identity is 57%.

Since autophosphorylation of the insulin receptor occurs only on tyrosine residues within the cytoplasmic domain of the β subunit, it was important to locate the position of each

EXPERIMENTAL PROCEDURES

RESULTS

Amino Acid Sequence of the Insulin Receptor of Mouse 3T3-L1 Adipocytes—To identify the site(s) of insulin-activated autophosphorylation within the β subunit of the insulin receptor of mouse 3T3-L1 adipocytes, it was necessary to know the amino acid sequence of receptor from this source. Therefore, cDNAs which encode the proreceptor were isolated and sequenced. A cDNA library prepared with mRNA from differentiated 3T3-L1 adipocytes (26) was screened both with a 3.5-kilobase human insulin receptor probe and a 700-base pair mouse insulin receptor probe. Twenty-one independent cDNA clones, representing different overlapping segments of the translated region of the mRNA, were isolated and the inserts sequenced from their 5' and 3' ends. In addition, the inserts from three of these clones (λIRc-2, λIRc-9, and λIRc-11), which together comprise the entire translated sequence of the proreceptor, were ligated at common restriction sites to generate two pBluescript clones: pET-IR, which contains the entire coding sequence and p2-9, which is the fusion product of the inserts of λIRc-2 and λIRc-9. pET-IR and a group of 5' exonuclease III deletion plasmids generated from plasmid p2-9 were sequenced using vector primers or synthetic oligonucleotide primers derived from sequences of several of the cDNA clones (see Fig. 4, Miniprint). The sequences obtained from these clones were assembled to generate the full-length nucleotide-coding sequence (see Fig. 5, Miniprint) for the mouse insulin receptor cDNA.

The deduced amino acid sequence of the mouse 3T3-L1 adipocyte insulin proreceptor is shown in Fig. 6 (see Miniprint), along with the corresponding sequence of the mouse insulin receptor. As in the case of the human proreceptor, the mouse proreceptor contains a 27-residue signal peptide. The overall amino acid sequence identity between the two receptors is 95%, with the α and β subunits exhibiting 97% and 94% identity, respectively. The positions of the 36 cysteine residues in the α subunit are totally conserved in both receptors, while 9 of 10 cysteines in the β subunit of the human receptor are conserved in the mouse receptor with the exception of cysteine 971, which is substituted by a serine residue. The number of relative positions of potential N-linked glycosylation sites are the same in both receptors, and the sequence of the COOH terminus of the α subunit, which contains the putative proteolytic cleavage site and gives rise to the α and β subunits, is totally conserved. The transmembrane sequences of the β subunit in both receptors are identical, and the intracellular domain, which contains the tyrosine kinase catalytic site, is highly conserved exhibiting 95% amino acid sequence identity. The intracellular segment of the β subunit, which begins at arginine 943 (941 in the human proreceptor) contains a consensus ATP-binding site (Gly-X-Gly-X-X-Gly; residues 993–998), 50-amino acid residues from the transmembrane domain. The β subunit also contains a lysine residue at position 1020 that is equivalent to lysine 1018 in the human proreceptor which has been shown to be essential for tyrosine kinase activity (31). The only region of significant sequence divergence between the mouse and the human receptors is at the NH2 terminus of the β subunit encompassing residues 728–735 (presumably an extracellular region), where the amino acid sequence identity is 57%.

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REFERENCES

1. The numbering system used in that reported for the human insulin receptor by Ulrich et al. (4).

2. Portions of this paper (including “Experimental Procedures” and Figs. 1–5 and 13–15) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Insulin Receptor Auto- and Substrate Phosphorylation

Effect of Insulin on the Kinetics of Receptor Autophosphorylation—Preliminary experiments were conducted (see "Experimental Procedures") to verify that the purified insulin receptor and its isolated β subunit were suitable for use in experiments to identify specific autophosphorylation sites required for the activation of protein substrate phosphorylation. It was established 1) that autophosphorylation of the 85-kDa β subunit is markedly activated by insulin (Fig. 1, Miniprint); 2) that the insulin receptor preparation does not contain active IGF-1 receptor; 3) that the Kₐ for insulin is the same (2-4 nM) for both autophosphorylation and RCAM-lysozyme phosphorylation; 4) that following autophosphorylation, the ³²P-labeled β subunit of the insulin receptor can be quantitatively recovered by immunoprecipitation and hydrophobic NH₂-terminal signal sequence (60% identity, Figure 2); 5) that the insulin receptor preparation does not contain active IGF-1 receptor; 6) that the autophosphorylation sites in the human insulin receptor (19) in the mouse proreceptor, these tyrosines are found at positions 962, 1148, 1152, 1153, 1318, and 1324 which are equivalent to tyrosines 960, 1146, 1150, 1151, 1316, and 1322 in the human insulin proreceptor.

Subunit of the mouse receptor contains 13 tyrosine residues, which corresponds to lysine 1018 in the HIR that is essential for tyrosine kinase activity (31). Both lysine 1020 representing the transmembrane spanning region in the MIR plus 3 basic residues which follow that region exhibit 100% identity to the HIR sequence.

The double dots (•) indicate identical amino acid residues. The conserved (●) and divergent (○) cytoplasmic tyrosines of the MIR and HIR are indicated. Also shown is a conserved lysine at position 1020 (*) in the MIR which corresponds to lysine 1018 in the HIR that is essential for tyrosine kinase activity (31). Both lysine 1020 and the Gly-X-Gly-X-Gly sequence (crosshatched box; residues 999-998 in MIR) comprise the conserved sequence (●) for an ATP-binding site. Based on analogies with the HIR (4), the mouse proreceptor also contains a ATP-binding site sequence (32) for an ATP-binding site. Based on analogies with the HIR (4), the mouse proreceptor also contains an ATP-binding site sequence (32) for an ATP-binding site. Based on analogies with the HIR (4), the mouse proreceptor also contains an ATP-binding site sequence (32) for an ATP-binding site.
polypeptide (Fig. 3, Miniprint) by two-dimensional nonreducing/reducing SDS-PAGE, contains only $^{32}$P phosphotyrosine with no detectable $^{32}$P phosphoserine or phosphothreonine (Fig. 2, Miniprint Supplement; and 8) that the model substrate, RCAM-lysozyme, is phosphorylated by the receptor exclusively on tyrosine residues (Fig. 3). On the basis of these results we concluded that the insulin receptor preparation was suitable for use in the autophosphorylation site analysis experiments described below.

When autophosphorylation of the receptor is conducted in the presence of insulin, there is an 8- to 10-fold stimulation in rate during the first 10 min of reaction relative to the basal rate in the absence of insulin (Fig. 7A). As is evident from the progress curve, autophosphorylation in the presence of insulin reaches an apparent maximum after approximately 30 min (Fig. 7A), whereas the maximal capacity of the receptor kinase to catalyze phosphorylation of RCAM-lysozyme is achieved within 5 min of autophosphorylation (Fig. 7B). Since insulin-stimulated autophosphorylation achieved only 60% of its maximum in 5 min, it is clear that full activation of the receptor tyrosine kinase does not require the maximal extent of autophosphorylation. These results are consistent with kinetic evidence obtained earlier in this laboratory (15) for activating and nonactivating components of autophosphorylation. Thus, at least half of the maximal activation of substrate phosphorylation occurs within the first minute of insulin-stimulated autophosphorylation, at which time autophosphorylation has reached approximately 37% of its maximum (see Fig. 7A).

The results illustrated in Fig. 7 show that in the absence of insulin, autophosphorylation approaches a plateau by 60 min having reached about 40% of the level achieved with insulin present (Fig. 7A). In contrast, the rate of substrate phosphorylation in the absence of insulin plateaued within about 15 min at which point it had achieved about 20% of the maximal insulin-stimulated rate. These findings validate previous suggestions (15) that the nature of autophosphorylation differs in the absence versus the presence of insulin. When the addition of insulin is delayed until autophosphorylation has proceeded for 15 min (without insulin), there is an immediate stimulation of autophosphorylation (Fig. 7A, at arrow) and an associated activation of substrate phosphorylation (Fig. 7B, at arrow). The progress curves for both activities and the half-times for reaching their maximal levels are similar to those obtained when insulin was added at zero time (Fig. 7).

**Autophosphorylation at Specific Sites in the β Subunit of the Receptor in the Absence and Presence of Insulin**—As an initial step in the identification of autophosphorylation sites required for the activation of catalysis of substrate phosphorylation, insulin receptor was incubated with [γ-$^{32}$P]ATP in the absence or presence of insulin after which the $^{32}$P-labeled β subunit was isolated by two-dimensional nonreducing/reducing SDS-PAGE (see “Experimental Procedures” and Fig. 3). The gel segments containing the $^{32}$P-labeled β subunit were exhaustively digested with trypsin and the resulting $^{32}$P phosphopeptides were separated by C$_4$ reverse-phase HPLC (Fig. 8). In this experiment, the phosphorylation reaction was allowed to proceed for 15 min since insulin-stimulated substrate phosphorylation reaches its maximum during this period of time (Fig. 7B).

In both the basal and insulin-stimulated cases, at least six discrete $^{32}$P phosphopeptide peaks (hereafter referred to as “sites”) were detected and designated p1, p2, p3, p4, p5, and p6, based on their order of elution during HPLC (Fig. 8). The first four peaks eluted, p1, p2, p3, and p4, have retention times of 13, 16, 20, and 29 min, respectively. Following the elution of p4, there is a variable region of decreasing radioactivity where two small peaks are occasionally detected (not shown). Peak p5 is eluted as a broad, heterogeneous peak, with a retention time of 54–58 min, while the last peak detected, p6, is eluted after 65 min. Although insulin-stimulated autophosphorylation is observed to some extent at all sites, the major stimulation occurs at sites p1, p2, and p3. It is apparent, however, that a significant fraction of the phosphorylation at all sites in the presence of insulin, represents insulin-independent autophosphorylation (see Fig. 8, upper
Insulin receptor was autophosphorylated for 15 min, either in the absence (top panel) or presence (bottom panel) of 1 μM insulin. Tryptic phosphopeptides from the [32P]-β subunit, prepared as described under "Experimental Procedures," were applied to a C8 Synchronpak reverse-phase column developed in 0.1% phosphoric acid/triethylamine, pH 6. Elution was initiated 5 min after injection with a gradient of isoproxy alcohol/acetonitrile 1:1 (dotted line) and a flow rate of 1.5 ml/min (see "Experimental Procedures"). Recovery of the 32P-label applied was 85% in both cases. The radioactive peaks were designated p1, p2, p3, p4, p5, and p6, according to their order of elution.

Size analysis of p1, p2, and p3 by HPLC size-exclusion chromatography and SDS-PAGE in 10-20% gradient gels revealed that these sites represent small phosphopeptides with apparent Mr values of 1,000-2,000 (results not shown). A similar analysis of the other HPLC peaks, p5 and p6, was considerably more difficult due to their insolubility and consequent poor recovery. However, the latter sites appear to correspond to heterogeneous mixtures of peptides with apparent Mr values in the 2,000-30,000 range, which probably represent incompletely digested β subunit.

Kinetic Correlation between the Activation of Substrate Phosphorylation and Autophosphorylation of Specific Sites in the Receptor’s β Subunit—The progression of autophosphorylation of specific sites on the receptor’s β subunit was followed kinetically during the period of greatest increase in insulin-stimulated substrate phosphorylation, i.e., during the first 60 s following insulin addition (Fig. 7B). During this initial phase of the activation process, about 60% of the maximal extent of activation of substrate phosphorylation occurs.

To minimize 32P-labeling of the autophosphorylation sites least relevant to the activation of substrate phosphorylation by insulin, the receptor was first preincubated for 15 min with unlabeled ATP in the absence of insulin. Under these conditions, the sites of autophosphorylation which contribute least to the activation (by insulin) of substrate phosphorylation would become occupied with unlabeled phosphoryl groups. To initiate autophosphorylation of sites associated primarily with the activation of substrate phosphorylation, both insulin and [γ-32P]ATP were added, and autophosphorylation of specific sites and the rate of substrate phosphorylation were monitored during the next 60 s (Figs. 9 and 10, respectively).

The kinetics of activation of substrate (RCAM-lysozyme) phosphorylation following autophosphorylation in the absence of insulin. Insulin receptor was autophosphorylated with ATP in the absence of insulin for 15 min. Insulin was then added (●, solid line) or not (○, dashed line), and the initial rate of substrate (100 μM RCAM-lysozyme) phosphorylation was measured at the times indicated (between 0 and 60 s) as described under "Experimental Procedures." It should be noted that further autophosphorylation, and hence activation, is blocked from the time of addition of RCAM-lysozyme. Data are from three independent experiments.

Insulin-stimulated 32P-labeling of tryptic phosphopeptides from receptor previously autophosphorylated with unlabeled ATP in the absence of insulin. To insulin receptor, previously autophosphorylated with unlabeled ATP in the absence of insulin (see Fig. 9), were added [γ-32P]ATP and insulin. After 10, 20, 30, and 60 s, the reaction was quenched and the 32P-β subunit was isolated and exhaustively trypsinized as described under "Experimental Procedures." [32P]Phosphopeptides were then separated by C8 reverse-phase HPLC under the conditions described in Fig. 8. The time of autophosphorylation in the presence of [γ-32P]ATP is indicated in the upper right corner of each HPLC elution profile.
phosphorylation capacity following insulin addition are shown in Fig. 9 (solid line). Upon the addition of insulin, there is an initial lag of 10–20 s in the activation of RCAM-lysozyme phosphorylation, which then increases abruptly during the next 40 s. By 60 s, the initial rate of substrate phosphorylation has reached nearly 60% of its maximal level. In contrast, if insulin is not added there is virtually no change in the initial rate of RCAM-lysozyme phosphorylation during the 60-s incubation with [γ-32P]ATP (Fig. 9, solid line).

The autophosphorylation of specific sites on the β subunit of the receptor was monitored concomitantly following the addition of insulin and [γ-32P]ATP in an experiment identical to that described above (Fig. 9) in which the activation of substrate phosphorylation was analyzed. The progress of autophosphorylation of the major autophosphorylation sites, analyzed by HPLC mapping of the 32P-labeled tryptic phosphopeptides derived from the insulin receptor, is shown in Fig. 10. The temporal relationship between the extent of autophosphorylation of each site and the extent of activation of substrate phosphorylation, is illustrated in Fig. 11. It is evident that the phosphorylation of site p1 (first panel) correlates best with the rate of activation of the receptor. However, it should be noted that phosphorylation at site p2 occurs faster than the activation of substrate phosphorylation during the first 30 s (Fig. 11, second panel) preceding the phosphorylation of site p1. The phosphorylation of p2 and p3 plateaus early and remains constant for the next 30 s when the rate of activation of substrate phosphorylation is increasing rapidly (from 24 to 58% of maximal activation; see also Fig. 9).

Finally, the kinetics of phosphorylation of p5 and p6 clearly depart from that of activation of substrate phosphorylation, although in the case of p6 it is rather difficult to obtain an unequivocal trend due to the great variability observed for this peak. However, in the experiments presented below, the phosphorylation of p5 and p6 are shown to occur independent of activation of substrate phosphorylation.

Based on these findings, it is evident that insulin-induced activation of the receptor kinase (for substrate phosphorylation) most closely correlates with autophosphorylation of site p1. It should be noted, however, that phosphorylation at other sites (p2, p3, and p6) precedes the phosphorylation at site p1, raising the possibility that activation of the receptor kinase may be the result of a series of concerted autophosphorylation events required for phosphorylation at site p1.

**Effect of Inhibiting Autophosphorylation at Sites p1 and p2 on Insulin-stimulated Substrate Phosphorylation**—To further explore the role of autophosphorylation sites p1 and p2 in the activation of substrate phosphorylation, the known inhibitory effect of substrate (i.e., RCAM-lysozyme) on autophosphorylation (15) was investigated. We previously showed that RCAM-lysozyme is both an excellent substrate for the fully activated (autophosphorylated) insulin receptor and a potent inhibitor of insulin-stimulated autophosphorylation (15). Thus, the addition of RCAM-lysozyme prior to (or with) insulin blocks autophosphorylation and, therefore, the activation of substrate phosphorylation.

The concentration dependence of inhibition of insulin-stimulated autophosphorylation and substrate phosphorylation by RCAM-lysozyme is shown in Fig. 12, A and B, respectively. In both cases, autophosphorylation was carried out for 5 min in the presence of insulin and increasing levels (0–100 μM) of RCAM-lysozyme. In Fig. 9B, substrate phosphoryla-

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6 To rule out the possibility that the lag phase was due to a mixing effect, an experiment was conducted in which the kinetics of receptor-catalyzed RCAM-lysozyme phosphorylation was followed at closely spaced time intervals (s). The rate of 32P incorporation into RCAM-lysozyme was found to be completely linear from 5 to 300 s. Moreover, when the receptor was allowed to equilibrate with 1 μM insulin before initiating autophosphorylation at zero time with [γ-32P]ATP (i.e., without a previous 15-min incubation with unlabeled ATP), the activation of substrate phosphorylation also underwent a 10–20 s lag, although in this case the contribution of the "basal" component was significant.
Insulin Receptor Auto- and Substrate Phosphorylation

Assignment of the Positions of Autophosphorylation Sites of the Insulin Receptor—From the primary structure of the insulin receptor of 3T3-L1 adipocytes (see Fig. 6), the amino acid sequences of all tyrosine-containing peptides that would be generated by complete digestion of the β subunit with trypsin can be predicted (Table I). Three of these tryptic peptides, T1, T3, and T5, can be eliminated as candidates for identity with p1, p2, and p3 on the basis of molecular weight. As indicated above, p1, p2, and p3 have molecular weights (M, = 1000–2000) much lower than those calculated for T1, T3, and T5. Since the quantities available of the tryptic phosphopeptides corresponding to autophosphorylation sites p1, p2, and p3 were insufficient for direct amino acid sequencing, an alternative approach was used. Given that phosphotyrosine is the only phosphoamino acid present in the β subunit of the insulin receptor that has undergone insulin-stimulated autophosphorylation (Fig. 2), it was possible to determine the position(s) of [32P]phosphotyrosine in autophosphorylation sites p1, p2, and p3 by Edman radiosequencing. With this information and knowledge of the amino acid sequence, the specific positions of these autophosphorylation sites within the β subunit were deduced.

Fig. 13 (Miniprint) shows the level of 32P release (derived from [32P]phosphotyrosine) at each cycle in gas-phase amino acid sequencing runs on each of the three [32P]phosphopeptides, i.e., p1, p2, and p3. It should be noted that the phosphotyrosine phenylthiohydantoin-derivative formed in the gas-phase sequenator is not efficiently released due to its insolubility in the extraction solvent which gives rise to the leaching of 32P observed in subsequent cycles (19). The results are expressed relative to the total amount of 32P radioactivity recovered in 20 cycles of Edman degradation. The patterns of

Fig. 12. Comparison of the inhibitory effect of RCAM-lysozyme on autophosphorylation, substrate phosphorylation, and specific sites of autophosphorylation. A, insulin receptor was autophosphorylated with 1 μM insulin for 5 min, in the presence of increasing concentrations of RCAM-lysozyme. The reaction was quenched and the incorporation of 32P into the β subunit was determined. The results are expressed relative to the maximal extent of autophosphorylation in the presence of insulin. B, receptor was autophosphorylated with unlabeled ATP, 1 μM insulin, and increasing concentrations of RCAM-lysozyme. After 5 min, a 10-μl aliquot was mixed with a saturating amount (100 μM) of RCAM-lysozyme and [γ-32P]ATP, in a total reaction volume of 50 μl. The reaction was then allowed to proceed for one additional min, and the incorporation of 32P into RCAM-lysozyme was determined. C, receptor was autophosphorylated with unlabeled ATP for 15 min, followed by the addition of [γ-32P]ATP and insulin, either in the absence or presence of 2 μM RCAM-lysozyme. The reaction was then continued for 60 s, terminated, and HPLC tryptic phosphopeptide maps generated as described in Fig. 8. Top panel, receptor autophosphorylated with insulin; bottom panel, receptor autophosphorylated with insulin plus RCAM-lysozyme. The radioactivity found in each peak was p1, 2,614 cpm versus 452 cpm; p2, 1,600 cpm versus 1,110 cpm; p3, 715 cpm versus 780 cpm; p5, 461 cpm versus 400 cpm; p6, 2,207 cpm versus 1,723 cpm, in the presence of insulin or insulin plus RCAM-lysozyme, respectively.
release of radioactivity for p1, p2, and p3 matched the predicted positions of tyrosine in two different tryptic peptides. For p1, there were significant increases in the release of radioactivity in cycles 3, 7, and 8, which correspond only to the predicted positions of tyrosine in peptide T4 (Table I). Similarly, site p2 also gave rise to the release of radioactivity at cycle 3, with an additional smaller release in cycle 7. This suggested that p2 and p1 represent the same peptide, T4, phosphorylated only at two of its tyrosine residues at positions 1318 and 1324, which correspond to positions 1316 and 1322 in the human insulin receptor. For p1, there were significant increases in the release of radioactivity in cycles 3, 7, and 8, which correspond only to the predicted positions of tyrosine in two different tryptic peptides.

Further Characterization of Autophosphorylation Sites p1, p2, and p3—The results of Edman radiosequencing of p1 and p2 suggested that both tryptic phosphopeptides represent the same domain of autophosphorylation (the region containing tyrosines 1148/1152/1153), which is phosphorylated to different extents. This could occur if the multiple tyrosyl residues within this domain underwent sequential phosphorylation. It was important, therefore, to determine whether p1 and p2 indeed represent the same phosphopeptide that is phosphorylated to different extents.

The initial resolution of p1, p2, and p3 by reverse-phase HPLC appeared to resolve domains of autophosphorylation but might not have yielded homogeneous phosphopeptides; therefore, each of the three phosphopeptides was subjected to further fractionation by ion-exchange chromatography. Charge heterogeneity within a phosphopeptide could not only result from differences in number of phospho~l groups but also from differences in the number of the COOH terminal basic amino acid residues arising from differential tryptic cleavage of pairs of basic amino acids (see Table I).

Fractions from the C, reverse-phase HPLC column containing each peak (p1 and p2) were separately pooled and subjected to anion-exchange chromatography on a Mono Q FPLC column (Pharmacia LKB Biotechnology Inc.). Both p1 and p2 gave rise to two [32P]phosphopeptides, designated p1A and p1B or p2A and p2B, as shown in Fig. 14 (Miniprint). As expected, both p1A and p1B were eluted after p2A or p2B, consistent with their higher phosphoryl group content (compare retention times in Fig. 14; p1A, 30 min and p1B, 34 min versus p2A, 21 min and p2B, 26 min).

As shown in Fig. 15 (Miniprint), secondary peptide mapping of these phosphopeptides by thin layer electrophoresis indicated that p1A and p1B correspond to two distinct homogeneous species which are considerably more acidic than p2A and p2B (compare lanes 1 and 2 with lanes 5 and 6 in Fig. 15). As expected from their order of elution from the Mono Q column, p1B is more acidic than p1A, to about the same extent as p2B is more acidic than p2A.

Since the amino acid sequence of the p1/p2 tryptic peptide which contains tyrosines 1148/1152/1153 includes only a single glutamic acid at position 1147 (interposed between tyrosine 1148 and tyrosines 1152/1153; see Table I, peptide

### Table I

| Peptide | Sequence |
|---------|----------|
| T1      | R KR QIQPMGLYASSNPYELASDVTPSVTVYVPDEWVR |
| T2      | R ELIQSFPGMVEGAK |
| T3      | R SLPDAENPGRPPTLQSM1QMTAEIADGMAYNLAK |
| T4      | R DAVTYDFVR K |
| T5      | K DGVFAASDDMSFGVVLWEITSLAEQVPQGLSNEQVQLK |
| T6      | K FWMDGYYLDPDNCPer |
| T7      | K DDLHPSPEVSSFYSEENK |
| T8      | K R TDHHPYTHMNGKK K |

The tyrosine-containing peptides that would be generated upon complete tryptic digestion of the cytoplasmic domain of the β subunit are shown (numbered T1 through T8) in order of their appearance in the primary structure of 3T3-L1 adipocyte insulin receptor (see Fig. 6). The positions of the tyrosine residues are indicated by the dots and tryptic cleavage sites by arrows. Restricted sites of tryptic digestion (due to a proline on the C-side), are also indicated (•).
**Insulin Receptor Auto- and Substrate Phosphorylation**

**TABLE II**

| Phosphopeptide* | Deduced structures and phosphorylation states of phosphopeptides p1, p2, and p3 |
|-----------------|---------------------------------------------------------------------------------|
| p1              | 1146 DIYETDYYRK ▲ ▲ ▲ 1155                                                   |
| p1A             | 3                                                                              |
| p1B             | 2                                                                              |
| p2              | 1146 DIYETDYYRK ▲ ▲ ▲ 1155                                                   |
| p2A             | 17                                                                   |
| p2A'            | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲                                                      |
| p2B             | 2                                                                              |
| p3              | 1316 RTYDEHIPYTHMNGGK ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ 1331                 |
| p3A             | 3                                                                              |
| p3B             | 2                                                                              |

o [32P]|Phosphopeptides, p1, p2, and p3, were initially resolved by reverse-phase HPLC. Secondary separation by Mono Q anion-exchange chromatography of p1, p2, and p3 gave rise to p1A and p1B, p2A and p2B, and p3A and p3B.

The arrowheads indicate tyrosine residues that are phosphorylated.

The monophosphorylated character of p2A is inferred (see "Results" for details).

T4), cleavage after this residue with staphylococcal V8 protease should generate two tyrosine-bearing peptide products. Determination of the extents of phosphorylation of these peptides, relative to each other, should indicate whether one, two, or all three tyrosines are phosphorylated. As expected, subdigestion of p1A and p1B with V8 protease yielded two discrete phosphopeptides, the most acidic of which exhibited the same electrophoretic mobility (see Fig. 1) in lanes 3 and 4 in Fig. 15). The other phosphopeptide product in each case behaved as a less acidic species than the corresponding parental phosphopeptide. Quantitation of the radioactivity associated with each phosphopeptide product revealed that their [32P] contents were in a 2:1 ratio, where ½ of the radioactivity corresponds to the least acidic phosphopeptide (see quantitation in legend to Fig. 15). Thus, this phosphopeptide corresponds to the COOH-terminal fragment bearing two phosphorylated tyrosines (positions 1152 and 1153) while the most acidic phosphopeptide represents the NH2-terminal fragment containing one phosphorylated tyrosine (position 1148). Taken together with the results of the Edman radiosequencing experiments, we conclude that p1 represents a triply tyrosine-phosphorylated form of the peptide containing tyrosines 1148/1152/1153. The occurrence of subspecies p1A and p1B is due to variable digestion by trypsin at the COOH terminus of this peptide, which would be expected to generate two products which differ by an additional lysine at the COOH terminus (Table I), and hence, differ in charge. It should be noted that limited subdigestion of p1A with carboxypeptidase B, generates a phosphopeptide with the same electrophoretic mobility as p1B, confirming that these phosphopeptides differ only by the presence of an additional COOH-terminal basic residue, in this case lysine (results not shown).

For the analysis of p2 by digestion with V8 protease, phosphopeptide p2B was used since it constituted the major component (by a factor of 2.5-fold) after purification by Mono Q anion-exchange chromatography (see Fig. 14). When p2B was digested with V8 protease, three widely separated phosphopeptides were produced whose [32P] contents were in the ratio 1:0.75:1 in descending order of mobility, as shown in Fig. 15 (lane 8). The most acidic phosphopeptide had the same mobility as the most acidic V8 protease digestion product of p1A or p1B, and represents the NH2-terminal fragment of the autophosphorylation domain, which contains tyrosine 1148. The intermediate-mobility [32P]phosphopeptide product behaved similarly to the least acidic V8 digestion product of p1A, comprising the COOH-terminal fragment of the same domain and bearing two [32P]phosphotyrosines (1152 and 1153). Finally, the least acidic phosphopeptide represented a new species not observed above which exhibited the lowest electrophoretic mobility of all the phosphopeptides described. We believe that this phosphopeptide corresponds to the COOH-terminal fragment of the autophosphorylation domain, which contains only one phosphorylated tyrosine (either 1152 or 1153; see Table II).

Our interpretation of these results is that the major species of p2, i.e. p2B, corresponds to the same peptide as p1, but is composed of a mixture of two doubly phosphorylated forms, one involving tyrosines 1148 and 1152 or 1153, the other involving tyrosines 1152 and 1153. In support of this interpretation is the fact that the ratio of phosphorylation ([32P]) of the most acidic to the least acidic [32P]phosphopeptide V8 protease digestion product is 1:1. This indicates that these products arose from the original peptide phosphorylated at tyrosines 1148 and 1152 or 1153. Thus, the other V8 protease digestion product, i.e. the intermediate mobility [32P]phosphopeptide, apparently resulted from cleavage of the peptide phosphorylated at the vicinal tyrosines 1152 and 1153. In addition, the fact that this product has the same mobility as the possibility that this [32P]phosphopeptide represents incompletely digested p2B is unlikely since digestion of p1A or p1B (which represent the same peptide) went to completion in the same experiment. Moreover, elution of this intermediate mobility [32P]phosphopeptide from the cellulose plates and redigestion with V8 protease did not generate cleavage products (results not shown).
the least acidic V8 digestion product of p1A suggests that the species comprising p2B represents the doubly phosphorylated counterpart of p1A (but not p1B), bearing two basic amino acid residues at the COOH terminus (arginine and lysine; see Table I). If this is the case, the only alternative explanation to account for the less acidic character of p2A and p2B is that the former represents a monophosphorylated form of the tyrosine 1148/1152/1153 domain, bearing two basic amino acid residues (arginine and lysine) at the COOH terminus, which would render it more basic than p2B. In contrast, the more acidic p2B represents a heterogeneous mixture of doubly phosphorylated forms of the same domain, as described above. Thus, limited subdigestion of p2A with carboxypeptidase B (Fig. 15, lane 7) removes one COOH-terminal basic amino acid residue (i.e. lysine, giving rise to a phosphopeptide with one phosphoryl group and only one basic amino acid (arginine) at the COOH terminus, shifting its mobility to that of p2B, which contains two phosphoryl groups and two basic COOH-terminal amino acid residues. Since p2A is a minor component of p2, further attempts were not made to verify its possible monophosphorylated character.

Finally, based on the ratio of the mass/charge contents of the V8 protease digestion products of p2B, the level of the doubly phosphorylated form involving tyrosine 1148 and 1152 or 1153 is approximately 2.7-fold higher (2/0.75) than the doubly phosphorylated form involving vicinal tyrosines 1152 and 1153. Taken together, these results suggest that activation of substrate phosphorylation, caused by insulin-stimulated autophosphorylation of the receptor, involves the random addition of three phosphoryl groups to tyrosines 1148, 1152 and 1153 in the β subunit.

A similar analysis was performed for the autophosphorylation domain represented by tryptic phosphopeptide p3, which contains tyrosines 1318 and 1324 near the carboxyl terminus of the β subunit (see Table I). As shown in Fig. 14, further fractionation of p3 by anion-exchange chromatography gives rise to two \[^{32}P\]phosphopeptides designated p3A and p3B, where p3A represents the major component by a factor of 2-3-fold (Fig. 14). When analyzed by thin-layer electrophoresis, these phosphopeptides behaved as homogeneous species differing in charge with p3A being less acidic than p3B (Fig. 15, lane 9 and 10), consistent with their order of elution from the Mono Q column (Fig. 14). Limited subdigestion of p3A with carboxypeptidase B shifted its mobility to that of p3B (Fig. 15, lane 11), suggesting that these species differ by one basic amino acid residue at the COOH terminus. It should be noted, however, that in this particular case charge heterogeneity might also derive from variable tryptic digestion at the aminoterminal end of this domain, where there are two potential sites of tryptic cleavage (lysine 1315 and arginine 1316; see Table I).

Digestion of p3A with V8 protease generated two \[^{32}P\]phosphopeptide products in an approximate ratio of 1:1 (Fig. 15, lane 12). The most acidic phosphopeptide product exhibited an electrophoretic mobility higher than its parent peptide (p3A) and corresponds to the amino-terminal fragment of the autophosphorylation domain containing a phosphorylated tyrosine at position 1316. The other product barely migrated from the origin and corresponds to the carboxyl-terminal fragment of the same domain, phosphorylated at tyrosine 1324.7 Were p2A the result of tryptic cleavage at arginine 1316, the amino-terminal fragment generated by V8 protease digestion would have the sequence Thr-(P)Tyr-Asp-Glu. This phosphopeptide would be expected to have a very similar charge/mass ratio (and presumably a comparably electrophoretic mobility) to the corresponding amino-terminal V8 protease digestion fragment from the tyrosine 1148/1152/1153 domain, whose sequence is Glu-Ile-(P)Tyr-Glu. However, the acidic V8 protease digestion product from p3A migrated as a less acidic species (Fig. 15, lane 12) than the corresponding product from p1A, p1B, or p2B (see above). These observations suggest that p3A results from tryptic digestion after lysine 1315 (and not arginine 1316), thus generating a product which contains an additional arginine residue at the NH₂ terminus (thereby rendering it more basic and reducing its electrophoretic mobility).

We conclude that p3 represents an autophosphorylation domain near the COOH terminus of the β subunit containing phosphorylated tyrosines 1318 and 1324. Hence, p3 would be expected to give rise to two \[^{32}P\]labeled V8 protease digestion products in a ratio of 1:1. The species p3A and p3B represent the same peptide with differing charge characteristics probably resulting from an additional amino-terminal arginine (in p3A) generated by incomplete tryptic digestion. This interpretation is consistent with the Edman radiosequence analysis of p3, where the higher release of \[^{32}P\]phosphotyrosine at cycles 3 and 9 (with respect to cycles 2 and 8; see Fig. 13), could reflect the higher abundancy observed for p3A (see Fig. 14). Table II summarizes the deduced structures and phosphorylation states of all the phosphopeptides described above.

**DISCUSSION**

There is now compelling evidence that insulin-stimulated autophosphorylation of the insulin receptor's β subunit is an essential step in the activation of substrate phosphorylation catalyzed by its intrinsic tyrosine kinase (6-10). Autophosphorylation has been found to occur at multiple sites in the β subunit and involves at least six tyrosyl groups (19, 23). Several approaches have been employed to characterize the specific site(s) required for insulin-induced activation of substrate phosphorylation. First, mutation of the insulin receptor at tyrosines 1150 and 1151 result in a reduction of insulin-stimulated autophosphorylation and substrate phosphorylation, both in *vitro* and in intact cells (20). Second, antibodies raised against a synthetic peptide corresponding to the region in the human receptor containing tyrosines 1146, 1150, and 1151, immunoprecipitated a CNBr-cleavage fragment of the β subunit whose autophosphorylation correlated with insulin-induced activation of substrate phosphorylation (21). Third, an antiphosphotyrosine antibody inhibits insulin-activated substrate phosphorylation by interrupting autophosphorylation of the tyrosine 1146/1150/1151 site at the doubly phosphorylated stage (22). Finally, preferential autophosphorylation at this site occurs during the activation of substrate phosphorylation induced by insulin, although autophosphorylation at tyrosine 953 and/or 960 also correlates well with the activation process (23).

In the present investigation, a kinetic approach was used to follow autophosphorylation at specific sites in the mouse insulin receptor during the initial, acute phase (i.e. 0–60 s) of insulin-induced activation of substrate phosphorylation. Since basal autophosphorylation (in the absence of insulin) contributes substantially to overall autophosphorylation in the presence of insulin without materially activating substrate phosphorylation (Fig. 7), special precautions were taken to minimize its contribution.

The tryptic phosphopeptide patterns (analyzed by HPLC) from the insulin receptor autophosphorylated in the absence

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4 These assignments were made on the basis of the calculated mass/charge ratio of each V8 protease digestion product, as compared to the relative mobility of known phosphopeptides with a fixed number of phosphoryl groups.
or presence of insulin were found to be qualitatively similar (Fig. 8), suggesting that in both cases common sites of autophosphorylation are involved. However, since the partial activation of substrate phosphorylation (~20%) that occurs in the absence of insulin plateaus by 10–15 min of basal autophosphorylation (Fig. 7), the contribution of this component can be minimized by preincubating the receptor with unlabelled ATP for 15 min before adding insulin and [γ-32P]ATP (Fig. 10). This step ensured prior occupancy of "basal" sites of autophosphorylation (with unlabelled phosphotyrosyl groups) which thereby permitted us to simultaneously track insulin-induced activation of substrate phosphorylation and autophosphorylation at the sites in the β subunit most relevant to the activation process.

The identification of the relevant autophosphorylation sites in the β subunit was facilitated by having available the complete amino acid sequence of the mouse 3T3-L1 adipocyte insulin receptor (Fig. 6). The major predicted structural features of this receptor are similar to those of the human insulin receptor, as might be expected from the high degree of amino acid sequence identity (~95%) between the two receptors. The intracellular domain of the β subunit possesses a Gly-X-Gly-X-Gly sequence (residues 993–998) upstream from a lysine residue at position 1028 (1018 in the human receptor) which is typical of the ATP-binding site in most kinases (32). In addition, at least 5 of the 13 tyrosine residues in the cytoplasmic domain of the β subunit in the mouse receptor correspond to autophosphorylation sites identified in the human (19) and rat insulin receptors (22). As in the human receptor, these residues in the mouse receptor are distributed between two distinct sites in the β subunit: tyrosines 1148, 1152, and 1153 (1146, 1150, and 1151 in the human insulin receptor) are clustered in a region considered to be part of the tyrosine kinase domain, while tyrosines 1318 and 1324 (1316 and 1322 in the human receptor) are located near the COOH terminus of the β subunit. This information, taken together with the results of Edman sequencing (Fig. 13) and secondary proteolytic digestion of tryptic [32P]phosphopeptides (Fig. 15) allowed us to locate the relevant phosphotyrosines within the β subunit of the receptor from mouse 3T3-L1 adipocytes.

Activation of the receptor by insulin is rapid. Thus, within 1 min after the addition of insulin, substrate phosphorylation is activated >50% of its maximal rate (Fig. 1B). This defined the narrow time window during which autophosphorylation occurs at sites most relevant to the activation process. Within the first 10–20 s of insulin addition, there is a substantial lag phase in the activation of substrate phosphorylation (Fig. 9). During this period, however, significant labeling (by [γ-32P]ATP) of tryptic phosphopeptides p2 and p3, but not p1, is observed (Figs. 10 and 11). Following this lag, i.e. between 20 and 60 s, activation of substrate phosphorylation increases abruptly following virtually identical kinetics to the labeling of phosphopeptide p1, while the rate of labeling of p2 and p3 have virtually reached a plateau (Fig. 11). Structural characterization of these phosphopeptides revealed that p1 and p2 are both derived from the tyrosine 1148/1152/1153 domain but differ in their extent of phosphorylation. Thus, p1 corresponds to the triply phosphorylated form of this domain, while p2 corresponds to the doubly phosphorylated form (Table II). Finally, p3 is derived from the tyrosines 1318/1324 domain, which lies near the COOH terminus of the β subunit (Table II). In contrast to p1, the kinetics of 32P-labeling of p3 do not correlate with the insulin-induced activation of substrate phosphorylation (Fig. 11).

These findings show that the tyrosine 1148/1152/1153 domain undergoes rapid insulin-stimulated stepwise autophosphorylation. In a preliminary step preceding the acute onset of activation of substrate phosphorylation, the doubly phosphorylated form of this domain (p2) accumulates (Fig. 10). In a subsequent step, this form undergoes further autophosphorylation giving rise to the triply phosphorylated form (p1). It is only the formation of the triply phosphorylated tyrosine 1148/1152/53 domain, however, that correlates with the kinetics of activation of receptor-catalyzed substrate phosphorylation. We believe that this event corresponds to the critical transition shown by White et al. (22) to be interrupted by an antiphosphotyrosine antibody. Our results provide direct kinetic proof that activation of substrate phosphorylation is proportional to accumulation of the triply (and not the doubly) phosphorylated state of the tyrosine 1148/1152/1153 domain.

Despite the evident precursor-product relationship between the doubly and triply phosphorylated tyrosine 1148/1152/1153 form of the receptor, the fraction of doubly phosphorylated form is still substantially detectable (Fig. 8) even after the receptor has become fully activated (Fig. 7B), i.e. after 15 min of autophosphorylation in the presence of insulin. This finding indicates that the doubly-phosphorylated form is not completely converted into the triply phosphorylated form, suggesting that these two forms (represented by p1 and p2) are both present at the end point of activation by insulin. Given the tetrameric (β-α-α-β) structure of the insulin receptor and the intramolecular character of autophosphorylation (17), these observations may be explained by an asymmetric mode of autophosphorylation in which the αββα tetramer has one β subunit with two phosphoryl groups in the tyrosine 1148/1152/1153 domain, while the other β subunit has three phosphoryl groups. In this view, double phosphorylation of the tyrosine 1148/1152/1153 domain occurs first in one β subunit and somehow triggers full phosphorylation of the corresponding domain in the contralateral β subunit, resulting in activation of substrate phosphorylation. It should be noted that White et al. (22) have suggested that there may be a control point at the transition between these two forms in the intact cell. Moreover, our V8 protease digestion analysis of the tryptic phosphopeptide p2 indicates that the doubly phosphorylated state of the tyrosine 1148/1152/1153 domain can assume two different isoforms, one phosphorylated at tyrosines 1148 and either 1152 or 1153 and another phosphorylated at vicinal tyrosines 1152 and 1153. The occurrence of these variants probably reflects the fact that this domain undergoes multiple phosphorylation in a nonordered sequence of tyrosine phosphorylation. Although the relative levels of these forms were not followed kinetically during the acute phase of the insulin-induced activation of the receptor, at least one, and possibly both forms accumulate rapidly during the early lag phase of the activation process (Fig. 9) because phosphopeptide p2 is detected at all time points (Fig. 10).

Thus, acute activation of catalysis may not reflect vis à vis autophosphorylation of a specific tyrosine residue in the tyrosine 1148/1152/1153 domain but rather may be the result of the complete autophosphorylation of all three tyrosines.

Additional evidence implicating the triply phosphorylated tyrosine 1148/1152/1153 domain in the activation of substrate phosphorylation was obtained through studies of the inhibition of autophosphorylation with the model substrate, RCAM-lysozyme. Experiments presented in this paper and previously (15, 16) indicate that the insulin-induced component of autophosphorylation and thus, of substrate phosphorylation are selectively blocked by RCAM-lysozyme in a
concentration-dependent manner (see Fig. 12, A and B). The major impact of RCAM-lysozyme upon the sites of autophosphorylation at tyrosines 953 and 950 in the human insulin receptor has also been found to correlate with the extent of kinase activation (23). The equivalent of these tyrosines in the COOH-terminal domain of the β subunit is not affected. Thus, RCAM-lysozyme disrupts insulin-stimulated autophosphorylation within the tyrosine 1148/1152/1153 domain as reflected by a dramatic shift in the ratio of the triply phosphorylated form (p3, Fig. 12C), while blocking the activation of substrate phosphorylation to essentially the same extent (82 versus 85%, respectively). In contrast, the accumulation of the doubly phosphorylated form is only partially inhibited, i.e. by 30%, and autophosphorylation at tyrosines 1318 and 1324 in the COOH-terminal domain of the β subunit is not affected. Thus, RCAM-lysozyme inhibits the extent of kinase activation (23). The equivalent of these human insulin receptor has also been found to correlate with the tyrosine kinase function of the receptor (33).

Two major observations indicate that autophosphorylation at tyrosines 1318 and 1324 (p3) in the COOH-terminal domain of the β subunit, does not play an essential role in the acute activation of catalysis induced by insulin, as previously suggested by Herrera and Rosen (21). First, the kinetics of 32P labeling of p3 and of the activation of substrate phosphorylation, are not appropriately correlated during the acute phase of activation by insulin (Fig. 11). Second, inhibition of insulin-stimulated autophosphorylation and substrate phosphorylation with RCAM-lysozyme did not affect the incorporation of 32P into p3 (Fig. 12C). It should be noted, however, that autophosphorylation within this domain, like that in the doubly phosphorylated form of the tyrosine 1148/1152/1153 domain (p2), also precedes the acute onset of insulin-induced activation of substrate phosphorylation, rapidly reaching a plateau by 30–30 s (Fig. 11). Thus, although autophosphorylation of the tyrosine 1318/1324 domain is not kinetically correlated with the activation of substrate phosphorylation, a possible prerequisite role for this process cannot be excluded. Nevertheless, this possibility seems unlikely in view of the fact that proteolytic removal of this domain does not affect the tyrosine kinase function of the receptor (33).

Autophosphorylation at tyrosines 953 and/or 960 in human insulin receptor has also been found to correlate with the extent of kinase activation (23). The equivalent of these tyrosines in the mouse receptor are represented by tryptic peptides T1 and T2, including [32P]phosphopeptides with a predicted mass of 2,000–30,000 (results not shown), it is possible that tryptic phosphopeptide T1 is a constituent of p6. As stated above (see “Results”), the great variability in the phosphorylation of p6 did not permit us to unequivocally correlate it with the activation of substrate phosphorylation, although it is barely inhibited by RCAM-lysozyme (i.e. by 15–20%). Thus, it is still possible that preliminary autophosphorylation in this domain is required for the subsequent activation of catalysis. In fact, Herrera et al. (34) have suggested a critical role for this site in the activation process because autophosphorylation and substrate phosphorylation are prevented with an antibody directed against this region of the β subunit, but only when added before initiating autophosphorylation with ATP. However, a recent report by White et al. (35) indicates that substitution of tyrosine 960 by site-directed mutagenesis had no effect upon the tyrosine kinase function of the receptor. Additional studies will be required to ascertain the role, if any, of autophosphorylation at tyrosines 955 and/or 962 in the activation process.

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**Insulin Receptor Auto- and Substrate Phosphorylation**

**Materials:**
- Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), calf intestinal alkaline phosphatase, V8 protease and cyanogen bromide were obtained from Boehringer Mannheim or Promega.
- T7 polymerase and dideoxynucleotide sequencing kits were obtained from S.S. Biotechnics of Pharmacia.
- The cDNA coding for human insulin receptor alpha chain was obtained from Dr. Eric Lore. 
- TPCK-treated trypsin was from Worthington.
- Radiolabeled insulin was obtained from Dainabot.
- Anti-rat liver insulin receptor antibody was generously provided by Dr. Ross Birnbaum.
- Anti-human insulin receptor antibody from Ascencos Signum was kindly provided by Dr. Philip Green (National Institute of Health).

**Methods:**
- The reaction mixture for measuring autophosphorylation of the beta subunit of the insulin receptor contained: 90 mM Hepes (pH 7.5), 1 mM EDTA, 1% Triton X-100 (v/v), and then they were heat-shocked to WGA-Sepharose for 90 min at 4°C, prepared as described (23).
- After repeating the matrix into a column, it was washed with 100 volumes of the same buffer and then with 100 volumes of the same buffer containing 1 M NaCl, and finally 100 volumes of 50 mM Hepes, 1 mM EDTA, 0.1% Triton X-100 at pH 6.9. Elution was achieved with the last buffer consisting of 0.3 M Na-azidoacetylamino. The eluates were sterile-filtered before use. The eluates were performed at pH 6.9 since this is the pH used in the phosphorylation reactions carried out subsequently. The chase was stored at 4°C.

**Measurement of Rate of Receptor Autophosphorylation and of Receptor-catalyzed Substrate Phosphorylation:**
- The reaction mixture for measuring autophosphorylation of the beta subunit of the insulin receptor contained: 90 mM Hepes (pH 6.9), 5 mM manganese acetate, 20 mM [γ-32P]ATP (40-80 cpm/pmol) and 4-10% of the receptor preparation. The final volume was 50 μl and incubations were conducted at 25°C. When present, insulin was added to a final concentration of 1 μM. The reaction was quenched after variable periods of time by addition of 25 μl of electrophoresis sample buffer consisting of 100 mM Tris (pH 6.6), 60 mM EDTA, 0.5% SDS, 10% glycerol and 0.05% bromphenol blue (w/v). The samples were applied without boiling onto 4% polyacrylamide slab gels. Electrophoresis was conducted at 150 V, and continued for 30 min after the dye front had passed off the corner of the gel thereby reducing residual γ-32P ATP and thus, background radioactivity in the gel. Unless otherwise indicated, gels were fixed and stained in 10% acetic acid, 20% methanol (v/v) and 0.05% Coomassie Brilliant Blue R250/water for 10 min. Destaining was done in the same solution without the dye, using two changes of 90% methanol and 10% acetic acid.

**Endogenous autophosphorylation and autophosphorylation (28, 29):**
- As shown in Fig. 3, [32P]labeled ATP activity detected by thin-layer chromatography on 3n-LI adipocytes was detected by thin-layer chromatography in the absence of insulin. 

**EXPERIMENTAL PROCEDURES**

**Supplementary Material:**
- The insulin receptor preparation was purified by affinity chromatography on WGA-Sepharose 4B and identified, based upon comparison with the gel segments, as that purified by the insulin receptor tyrosine kinase. 

**Kinetic Correlation of Roles of Receptor Autophosphorylation and of Receptor-Mediated Substrate Phosphorylation.**
- The reaction mixture for measuring autophosphorylation of the beta subunit of the insulin receptor contained: 90 mM Hepes (pH 6.9), 5 mM manganese acetate, 20 mM [γ-32P]ATP (40-80 cpm/pmol) and 4-10% of the receptor preparation. The final volume was 50 μl and incubations were conducted at 25°C. When present, insulin was added to a final concentration of 1 μM. The reaction was quenched after variable periods of time by addition of 25 μl of electrophoresis sample buffer consisting of 100 mM Tris (pH 6.6), 60 mM EDTA, 0.5% SDS, 10% glycerol and 0.05% bromphenol blue (w/v). The samples were applied without boiling onto 4% polyacrylamide slab gels. Electrophoresis was conducted at 150 V, and continued for 30 min after the dye front had passed off the corner of the gel thereby reducing residual γ-32P ATP and thus, background radioactivity in the gel. Unless otherwise indicated, gels were fixed and stained in 10% acetic acid, 20% methanol (v/v) and 0.05% Coomassie Brilliant Blue R250/water for 10 min. Destaining was done in the same solution without the dye, using two changes of 90% methanol and 10% acetic acid.

**Endogenous autophosphorylation and autophosphorylation (28, 29):**
- As shown in Fig. 3, [32P]labeled ATP activity detected by thin-layer chromatography on 3n-LI adipocytes was detected by thin-layer chromatography in the absence of insulin.

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3. J., M., A., and L., D., 1985. The insulin receptor preparation was purified by affinity chromatography on WGA-Sepharose 4B and identified, based upon comparison with the gel segments, as that purified by the insulin receptor tyrosine kinase. 

**FIGURE 1.** Insulin-stimulated autophosphorylation and substrate phosphorylation by the mouse insulin receptor. Phosphorylation reactions were conducted at pH 6.9 in 50 mM Hepes buffer, containing 0.15 Tris X-100 and 3 μg of the insulin receptor partial, 5 mM magnesium acetate, and 20 μM [γ-32P]ATP. Reactions were incubated with [γ-32P]labeled insulin for 5 min and were terminated by the addition of dephosphorylating sample buffer. [32P]labeled phosphoproteins were resolved by 7%-15% gradient SDS-PAGE. Reactions without insulin were run in lanes 1, 4, and 7. Reactions with 1 μM insulin - lanes 2, 3, 6, and 8. Reaction without IGF-I - lane 9 and with IGF-I - lane 10. The model substrate, RCM-lysophosphatidyl ethanolamine, was added at 100 μM after 5 min of autophosphorylation and the reaction continued for additional min - lanes 3, 6. The positions of the β-subunit (β) and RCM-lysophosphatidyl ethanolamine (RLC) are indicated. 

- It should also be noted that two different antibody preparations (anti-serum to insulin receptor and an anti-human insulin receptor antibody) were successfully immunoprecipitated the [32P]labeled 95 kDa β-subunit (as judged by SDS-PAGE and autoradiography) derived from the [32P]labeled insulin receptor. Thus, based on these findings, it was concluded that the insulin receptor preparation was suitable for autophosphorylation site analysis.

**Phosphoamino acid Analysis of [32P]labeled Insulin Receptor and RCM-lysophosphatidyl ethanolamine.** [32P]labeled insulin receptor β-subunit or RCM-lysophosphatidyl ethanolamine were located by autoradiography of [32P]labeled 95 kDa β-subunit (as judged by SDS-PAGE and autoradiography) derived from the [32P]labeled insulin receptor. 

**FIGURE 2.** Phosphoamino acid analysis of [32P]labeled β-subunit and RCM-lysophosphatidyl ethanolamine. The [32P]labeled insulin receptor β-subunit or RCM-lysophosphatidyl ethanolamine were located by autoradiography of [32P]labeled 95 kDa β-subunit (as judged by SDS-PAGE and autoradiography) derived from the [32P]labeled insulin receptor. Thus, based on these findings, it was concluded that the insulin receptor preparation was suitable for autophosphorylation site analysis.

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Isolation of the Insulin Receptor. To ensure isolation of the insulin receptor’s \( \beta \)-subunit, advantage was taken of the receptor’s unique \( \alpha \)-in-\( \beta \) oligomeric structure which is stabilized by covalent inter-subunit disulfide bonds. Thus, by two-dimensional non-reducing/SDS-PAGE, the receptor migrates in a \( \approx 110 \) kDa oligomeric species in the first (non-reducing) dimension (Fig. 1) and as \( \approx 130 \) kDa and \( 95 \) kDa \( \beta \)-subunits, respectively, in the second (reducing) dimension (Fig. 2). Following variable periods of autophosphorylation, the mixture was quenched as described above, except that the sample electrophoresis buffer \( \text{H}_{2}\text{O} \) did not contain DTT. Separation in the first dimension was accomplished with \( 5 \% \) acrylamide slabs gels. In preparation for the second dimension, the gels were excised from the glass tubes, and equilibrated for 9 min at room temperature with electrophoresis sample buffer containing 20 mM DTT. The second dimension was done in 8% acrylamide slabs gels, and the position of the \( \beta \)-subunits was located by autoradiography of the wet, unfixed gel. A typical \( \approx \) 9-kDa protein band is illustrated in Fig. 3. The migration of the insulin receptor in the first dimension is the result of its oligomeric structure (MW \( \approx 410 \) kDa), which is readily separated from contaminating \( \beta \)-subunit phosphoryrion in the \( 90-100 \) kDa range (see Fig. 3).

**FIGURE 3.** Isolation of the \( \beta \)-subunit by two dimensional non-reducing/reducing SDS-PAGE. An autoradiograph of the precipitate was conducted in the presence of \( \gamma \)-[\( \text{H} \)]-ATP as described in Fig. 1, and then quenched by the addition of electrophoresis sample buffer without DTT. The \( \beta \)-phosphoprotein was separated by non-reducing SDS-PAGE, followed by reducing SDS-PAGE as described in Experimental Procedures. The migration of molecular weight standards is shown on the left.

**Molecular Cloning and Sequencing of the cDNA Encoding the Mouse Insulin Receptor.** A cDNA library (>10⁶ clones in L22AP/Stratagene) prepared with poly(A) mRNA from differentiated 3T3-L1 adipocytes (26) was screened by plaque hybridization. \( ^{2} \text{H} \)-labeled probes used to screen this library, were generated from either a \( 3.5 \) kb EcoRI cDNA fragment (probe A) of the human insulin receptor (kindly provided by Dr. Y. Ehrlich and W. Rutter, Univ. of California, San Francisco) or a \( 500 \) bp PvuII cDNA fragment (probe B) of the mouse insulin receptor. The PvuII fragment was isolated from a mouse placenta cDNA library screened with a \( 230 \) bp SnaBI fragment derived from the 5’ end of a \( 750 \) bp mouse placenta cDNA (generously provided by Dr. S. Seino and D. Simon, Univ. of Chicago), encoding amino acids 46-205 of the \( \alpha \)-subunit. cDNA inserts of positive XZAP plasmid clones were excised to generate pBluescript phage subclones according to the protocol supplied by Stratagene.

Dissected phagotrophic cDNAs were sequenced by the chain termination method of Sanger et al. (27) using \( ^{35} \text{P} \)-labeled dNTPs, T7 polymerase, synthetic oligonucleotide primers and Sequenase (U. S. Biochemicals).

Twenty-one independent cDNA clones were isolated, sequenced from their 5’ and 3’ ends, and aligned with the human insulin receptor sequence (14). Phagotrophic cDNA from cDNA clone XM-2-2 digested with BglII and HindIII, was ligated to the \( 1.6 \) kb BglII/HindIII fragment of phagotrophic clone XM-2-9 to generate plasmid p3-9 which encompasses the entire coding region for the \( \beta \)-subunit and 235 of the coding region (5'-terminal for the \( \beta \)-subunit; Fig. 4, Appendix). The \( 3.3 \) kb EcoRI insert of phagotrophic XM-2-11 (reverse orientation, encompassing the C-terminal region of the \( \beta \)-subunit) was ligated back into linearized pBluescript to generate the reverse orientation plasmid p7'. The \( 1.4 \) kb XhoI fragment of p3-7 was ligated to XhoI-linearized p3-9 to generate plasmid pET-8R which contains the entire coding region of the mouse insulin receptor (Fig. 4, Appendix). pET-8R and 3’ exonuclease III digestions plasmids generated from p3-9 (Stragagram Blueprint Eva/Mang protocol), were sequenced using vector primers or synthetic oligonucleotide primers derived from the sequence of several cDNA clones. The full-length nucleotide coding sequence was generated from the assembled overlapping cDNA sequences obtained (see Fig. 5, Appendix) and was translated to generate the complete amino acid sequence of the precursor (Fig. 6).

**FIGURE 4.** Schematic representation of cDNA’s used to sequence the mouse 3T3-L1 adipocyte insulin receptor. A schematic representation of the mouse 3T3-L1 adipocyte insulin receptor/cDNA is shown at the top. The open boxes correspond to the translated region of the receptor which gives rise to the mature \( \alpha \)-and \( \beta \)-subunits. The cross-hatched box represents the region which encodes the signal peptide. Probes A and B were used to screen a ZAP/Stratagene cDNA library from which 21 positive XZAP clones were obtained. cDNA inserts from 3 of the ZAP clones, whose sequences overlap, were excised and incorporated into pBluescript phagotrophic clones XM-2-2, XM-2-9 and XM-2-11. p3-9 was constructed by ligation of the inserts from XM-2-2 and XM-2-9 or a common (BglII) restriction site. pET-8R was constructed by ligation of the inserts from p3-9 and XM-2-11 and a common (XhoI) restriction site. pET-8R and a family of 3’ exonuclease III deletions and wild-type and 5’ and 3’ ends of 21 XZAP positive clones were sequenced using vector or synthetic oligonucleotide primers. The arrows below the scheme indicate the regions from which cDNA sequences were obtained. See Experimental Procedures for details.
| 40 | 80 | 120 | 160 | 200 | 240 | 280 | 320 | 360 | 400 | 440 | 480 | 520 | 560 | 600 | 640 | 680 | 720 | 760 | 800 |
|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Insulin Receptor Auto- and Substrate Phosphorylation** |

**FIGURE 5.** Nucleotide sequence of the mouse 3T3-L1 adipocyte insulin promoter cDNA. The nucleotide sequence of pH-cR cDNA contains a 4156 bp open reading frame which encodes the 1372 amino acid mouse 3T3-L1 insulin receptor (MR) residue at methionine +1. The MR cDNA (nucleotides K241) exhibits 97.2% nucleotide sequence identity with respect to the corresponding region of the human insulin receptor (HM) cDNA H. See Experimental Procedures for details.
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Figure 13. Release of $^{32}$P radioactivity during Edman degradation of $^{32}$P phosphorylated p1, p2, and p3. $^{32}$P phosphorylated p1, p2, and p3 were prepared by reverse-phase HPLC from receptor autophosphorylation for 15 mins in the presence of insulin, as described in Fig. 8. The HPLC fractions containing these phosphorylated peptides were lyophilized and suspended in 10 μl of 50% acetic acid/sodium acetate/water (1:1:1). Each sample was subjected to gas-phase automated amino acid Edman sequencing and $^{32}$P radioactivity in the eluate from each cycle was determined by scintillation counting (see Experimental Procedures). The unextracted two-stage cycles in which $^{32}$P phosphorylation would be released if the tyrosine residues of the phosphorylated peptides analyzed correspond to those of the tryptic peptides shown in the upper portion of each panel. The results are expressed relative to the total amount of $^{32}$P radioactivity recovered after 20 cycles of Edman degradation of each phosphorylated peptide (p1: 48,337 cpm applied and 4,905 cpm recovered, p2: 24,061 cpm applied and 1,397 cpm recovered, p3: 21,250 cpm applied and 2,052 cpm recovered).

Figure 14. Further purification of $^{32}$P phosphorylated p1, p2, and p3 by Mono Q anion-exchange chromatography. Fractions from C₅ reverse-phase HPLC (see Fig. 8) containing $^{32}$P-labeled peaks p1, p2, and p3 were pooled separately and applied directly into a Mono Q column (Pharmacia) equilibrated with 0.02 M ammonium bicarbonate pH 7.8. The tryptic peptides were eluted with a 0.02-1 M linear gradient (dotted line) of ammonium bicarbonate over 60 mins and radioactivity determined by a Beckman γ-counter. In each case, peaks were designated with the suffix A or B according to their order of elution and are indicated by the arrows. Retention time: p1A, 30 mins; p1B, 34 mins; p2A, 21 mins; and p2B, 26 mins; p3A, 22 mins; and p3B, 28 mins. Overall recovery was 70%. The chromatograms are identified in the upper left corner.