Two-dimensional Phosphopeptide Analysis of the Autophosphorylation Cascade of a Soluble Insulin Receptor Tyrosine Kinase

THE TYROSINES PHOSPHORYLATED ARE TYPICAL OF THOSE OBSERVED FOLLOWING PHOSPHORYLATION OF THE HETEROTETRAMERIC INSULIN RECEPTOR IN INTACT CELLS*

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A soluble derivative of the human insulin receptor cytoplasmic domain, as expressed in insect cells via a Baculovirus vector, is an active protein-tyrosine kinase. In the present study, we find that three forms of the enzyme (48, 43, and 38 kDa) can be partially purified by MonoQ fast protein liquid chromatography. Two-dimensional thin layer phosphopeptide mapping reveals that the 48-kDa enzyme undergoes a rapid autophosphorylation on the same tyrosines (residues 1158, 1162, 1163, 1238, and 1334) that have previously been shown to be major autophosphorylation sites on the native insulin receptor β-subunit in intact cells. Furthermore, the 48- and 43-kDa proteins are phosphorylated on serine residues by a serine kinase(s) that copurifies through MonoQ fast protein liquid chromatography. Tyrosine autophosphorylation sites 1238 and 1334 and virtually all serine phosphorylation sites are absent in the 38-kDa kinase. Partial tryptic proteolysis of the 48-kDa kinase generates a core 38-kDa enzyme that undergoes autophosphorylation almost exclusively on tyrosines 1158, 1162, and 1163. Phosphorylation of these tyrosine residues occurs in a cascade manner analogous to that found in the intact insulin receptor β-subunit.

The mature insulin receptor exists as a transmembrane heterotetrameric αβ2γ2 glycoprotein (Kahn et al., 1985; Zick, 1989). The extracellularly disposed α-subunits bind the polypeptide hormone insulin and are connected via disulfide bonds to the transmembrane β-subunits. The cytoplasmic two-thirds of the β-subunit harbors a tyrosine-specific protein kinase that is stimulated upon insulin binding. Although several putative substrates have been identified for this kinase in intact cells, their significance to the mechanism of insulin signaling remains obscure. Indeed, the β-subunit itself may serve as the most important physiological substrate for the tyrosine kinase, as autophosphorylation both activates the tyrosine kinase toward exogenous substrates (Rosen et al., 1983; Yu and Czech, 1984, 1986; Klein et al., 1986) and promotes a conformational change in the β-subunit that exposes specific epitopes to recognition by antisera (Herrera and Rosen, 1986; Perlman et al., 1989).

In intact cells, insulin promotes a very rapid rise in the autophosphorylation of the insulin receptor β-subunit on tyrosines 1158, 1162, 1163, 1238, and 1334 (Tavare et al., 1988; Tornqvist et al., 1988; White et al., 1988). One or more tyrosines (residues 965, 972, and 984) close to the transmembrane domain of the receptor may also serve as sites of further autophosphorylation (Tornqvist et al., 1987; Tavare and Denton, 1988). In intact cells the rise in tyrosine autophosphorylation is followed, after a short lag, by a rise in serine and/or threonine phosphorylation of the β-subunit (White et al., 1985; Fang et al., 1985). In addition, an insulin-stimulated protein serine-kinase has been shown to copurify with the insulin receptor through chromatography on both wheat germ lectin-Sepharose (Ballotti et al., 1986; Smith et al., 1988; Lewis et al., 1990) and insulin-Sepharose (Lewis et al., 1990). However, the role of insulin-stimulated serine and threonine phosphorylation in insulin receptor signaling are not known.

The cytoplasmic protein-tyrosine kinase domain of the human insulin receptor (residues 959–1355) has been expressed as a soluble protein (401 amino acids, M, 45,297) in insect Sf9 cells by the use of a Baculovirus vector (Ellis et al., 1988). For studies of such a soluble derivative to be relevant to the function of the kinase in the intact and membrane-associated receptor, the soluble enzyme must exhibit functional properties characteristic of those of the native receptor kinase. As previously described, the soluble kinase is recognized by a panel of anti-insulin receptor monoclonal antibodies reactive toward conformation-dependent epitopes of the β-subunit cytoplasmic domain, exhibits insulin-independent autophosphorylation (apparently in trans between enzyme monomers), and phosphorylates a number of synthetic peptide substrates on tyrosine residues (Ellis et al., 1988; Cobb et al., 1989). The stoichiometry of tyrosine autophosphorylation of the soluble insulin receptor tyrosine kinase domain was shown to be 4 to 5 mol of phosphate/mol of enzyme (Cobb et al., 1989). In the present study we now show that this phosphate is incorporated at sites which are entirely typical of those seen with the intact heterotetrameric insulin receptor phosphorylated in intact cells.
Phosphorylation Sites of a Soluble Insulin Receptor Kinase

MATERIALS AND METHODS

Phosphorylation of the Soluble Insulin Receptor Protein-Tyrosine Kinase—Phosphorylation of the soluble enzyme on a preparative MonoQ FPLC column was performed as described (Cobb et al., 1989). Fractionations eluted from the column were stored at 4°C with little detectable loss in enzyme activity for up to 3 months following purification. In some experiments the soluble kinase (which migrates as a 48-kDa protein on SDS-PAGE) was treated with trypsin (20:1 ratio by weight of kinase to trypsin) for 10 min at 25°C to generate a 38-kDa species which was separated from trypsin by chromatography on soybean trypsin inhibitor-Sepharose (Pierce Chemical Co.) and further purified by MonoQ FPLC chromatography.

Protein Kinase Reactions—Fractions (~5 μg of protein) eluted from the MonoQ column were preincubated for 15 min at 30°C in a final volume of 50 μl containing 130 mM Hepes, pH 7.5, 4 mM MgCl₂, 0.3 mM-EDTA, 100 μM/ml bovine serum albumin, and 20 μM ATP. Incubation was continued for the indicated time periods at 30°C with 250 μM [γ-32P]ATP (500–1000 cpm/pmol). Reactions were terminated with sample buffer (Laemmli, 1970) and heating for 5 min at 100°C. Samples were submitted to SDS-PAGE (10% polyacrylamide gels; Laemmli, 1970), and the 32P-labeled kinase was electroeluted from the gel and precipitated with acetone as described (Tavare and Denton, 1988).

Phosphoamino Acid Analysis—Tryptic phosphopeptides were resolved on a preparative scale MonoQ anion exchange FPLC column. Fractions were incubated with [γ-32P]ATP and analyzed by SDS-PAGE. Autoradiography of the resulting gel (Fig. 2a) reveals a series of three major species of soluble insulin receptor tyrosine kinase, at 48, 43, and 38 kDa. When each of these species were analyzed for phosphoamino acid content (Fig. 2b), the 38-kDa kinase was found to be predominantly phosphotyrosine, with less than 5% phosphoserine. For the 43- and 48-kDa species, phosphotyrosine to phosphoserine ratios of 1:1 and 2:1 were observed, respectively. It should be emphasized that these ratios are purely a qualitative assessment of the relative amounts of phosphoserine to phosphotyrosine, as phosphoserine has a greater stability during acid hydrolysis than phosphotyrosine (Cooper et al., 1983).

Phosphopeptide maps of the 38-, 43-, and 48-kDa kinases demonstrate that the three major insulin receptor tyrosine autophosphorylation sites (residues 1158, 1162, and 1163) are recovered as a family of five phosphopeptides (general sequence DIYEDTDYRK), which are mono- (C1), bis- (B2 and B3), or tris- (A1 and A2) phosphorylated and cleaved by trypsin at arginine 1165 and either arginine 1164 (C1, B1, and A2) or lysine 1165 (B2 and A1). Tyrosine 1328 and 1334 are recovered as a single bis-phosphopeptide (sequence SYEHEHIP-YTHMNGGK) that migrates as peptide B1. Additional tyrosine autophosphorylation sites (probably residues 965, 972, and 984) migrate as peptides C1', C2, and C3 and are most readily observed in human insulin receptor phosphorylated in vitro in the presence of insulin and [γ-32P]ATP (Tavare and Denton, 1988). Serine and threonine residues phosphorylated in intact cells migrate as peptides S1 and T, respectively (Tavare et al., 1988).

RESULTS AND DISCUSSION

Tryptic digestion of the human insulin receptor β-subunit phosphorylated in intact cells reveals a complex pattern of phosphopeptides, as depicted by the cartoon in Fig. 1. The assignment of these phosphopeptides has been previously described (Tavare and Denton, 1988; Tavare et al., 1988) and is briefly summarized in the legend to Fig. 1.

Using the two-dimensional phosphopeptide mapping technique, we determined the sites of tyrosine autophosphorylation of the soluble insulin receptor tyrosine kinase expressed in Sβ cells. Forty h post-infection, cells were harvested, lysed, and fractionated using a preparative scale MonoQ anion exchange FPLC column. Fractions were incubated with [γ-32P]ATP and analyzed by SDS-PAGE. Autoradiography of the resulting gel (Fig. 2a) reveals a series of three major species of soluble insulin receptor tyrosine kinase, at 48, 43, and 38 kDa. When each of these species were analyzed for phosphoamino acid content (Fig. 2b), the 38-kDa kinase was found to be predominantly phosphotyrosine, with less than 5% phosphoserine. For the 43- and 48-kDa species, phosphotyrosine to phosphoserine ratios of 1:1 and 2:1 were observed, respectively. It should be emphasized that these ratios are purely a qualitative assessment of the relative amounts of phosphoserine to phosphotyrosine, as phosphoserine has a greater stability during acid hydrolysis than phosphotyrosine (Cooper et al., 1983).

Phosphopeptide maps of the 38-, 43-, and 48-kDa kinases demonstrate that the three major insulin receptor tyrosine autophosphorylation sites (residues 1158, 1162, and 1163, i.e. peptides C1, B2, B3, A1, and A2 in Fig. 3, a–c) are phosphorylated in all three species. A phosphopeptide migrating just below the dinitrophenyl lysine standard (designated D in...
48- and 43-kDa kinases have been removed to generate the 38-kDa species, most likely as the result of proteolytic cleavage.

Two additional soluble derivatives of the insulin receptor tyrosine kinase, also expressed in insect Sf9 cells by the use of a *Baculovirus* vector, have been previously reported by others (Herrera et al., 1988; Villalba et al., 1989). In contrast to the results of the present study, these two soluble kinases, which differ only in their amino-terminal sequences (MHAI DG::: versus MRRQPDG:::; note that the amino terminus of our 48-kDa kinase is MRPMG:::, where the G of all three sequences corresponds to glycine 959 of the wild-type receptor) were reported to exhibit atypically high levels of autophosphorylation in their amino-terminal domains (greater than 40% of the total $^{32}$P incorporated). Furthermore, up to 40% of the total $^{32}$P incorporated was attributed to the carboxyl-terminal domain, with less than 20% incorporated into tyrosines 1158, 1162, and 1163. While it is possible that the very different distribution of phosphorylation sites observed could be due to isolation of the enzyme with differing levels of phosphate content (hence a difference in the extent of occupation of sites with phosphate prior to incubation with [$\gamma$-$^{32}$P]ATP), it is also likely a result of the use of different analytical methods. Rather than using direct analysis of phosphorylation sites by peptide mapping, immunoprecipitation of $^{32}$P-labeled tryptic peptides with polyclonal anti-peptide antisera was employed (Herrera et al., 1988; Villalba et al., 1989), a method which we have found unsatisfactory due to inefficient immunoprecipitation of very small amounts of phosphopeptide by such sera.

Considerable amounts of phosphoserine were found in the 48- and 43-kDa kinase species (Fig. 2b). When the tryptic phosphopeptide maps were examined, peptide S1 (containing predominantly phosphoserine, data not shown) was present in the 48- (Fig. 3c) and 43-kDa (Fig. 3b) kinases, but not the 38-kDa kinase (Fig. 3a). This is consistent with the almost complete absence of detectable phosphoserine in the 38-kDa kinase as assessed by phosphoamino acid analysis (Fig. 2b). Peptide S1 has a very similar mobility to the phosphoserine containing tryptic peptide derived from native insulin receptor $\beta$-subunits isolated from insulin-treated and $^{32}$P-labeled cells (Fig. 1, see Tavare et al., 1988). The combined absence of peptides B1 (carboxyl-terminal tyrosines 1328 and 1334) and S1 from the 38-kDa kinase suggests that the serines phosphorylated in peptide S1 may also be derived from the carboxyl terminus of the $\beta$-subunit. This would be consistent with the proposal of others (Lewis et al., 1990), who have identified serines 1305 and 1306 as potential sites of serine phosphorylation of the insulin receptor $\beta$-subunit.

The 43-kDa kinase (Fig. 3b), but not 48-kDa kinase (Fig. 3c), also possesses a highly negatively charged phosphopeptide S2, which contains exclusively phosphoserine (data not shown). Its presence in the 43-kDa kinase certainly accounts for the greater apparent ratio of phosphoserine to phosphotyrosine in the 43-kDa kinase (1:1) compared with the 48-kDa kinase (1:2, see Fig. 2b). It is not clear at present why peptide S2 is not observed in the 48-kDa kinase. One possibility is that removal of a 5-kDa peptide fragment from the 48-kDa kinase exposes previously inaccessible serine(s) for phosphorylation.

We have not previously observed phosphopeptide S2 in maps from intact insulin receptors. However, its absence in the 38-kDa kinase suggests that the serines phosphorylated on peptide S2 may also reside in the carboxyl terminus of the kinase. Indeed a prominent $^{32}$P-labeled peptide of low molecular mass appears at the dye front of lanes 1–3 of Fig. 2a, the
to confirm this by either peptide mapping or phosphoamino acid analysis. Thus, we have no direct information concerning the precise location of the serine residues modified in the primary amino acid sequence. The identities of the kinase(s) responsible for their phosphorylation and their relationship to the previously non-phosphorylated population of kinase molecules undergo a slower rate of autophosphorylation (Fig. 4, c versus a). Similarly, we have also found that serine (and threonine) phosphorylation of the native insulin receptor

amount of which closely parallels that of the $^{32}$P-labeled 38kDa kinase present in these fractions. It is very likely that this represents the carboxyl terminal fragment released from the 48- and/or 43-kDa kinases during the formation of 38-kDa kinase. Furthermore, this fragment may still serve as a substrate for either the soluble tyrosine kinase or serine kinase(s). However, due to its small size, we have been unable to confirm this by either peptide mapping or phosphoamino acid analysis. Thus, we have no direct information concerning the precise location of the serine residues modified in the primary amino acid sequence. The identities of the kinase(s) responsible for their phosphorylation and their relationship to insulin-stimulated insulin receptor serine kinases in mammalian cells remain intriguing.

Tyrosine autophosphorylation of the 48-kDa soluble kinase is rapid (Fig. 4). Within 5 min of incubation with $[\gamma-^{32}\text{P}]$ATP, there is an immediate appearance of the tris-phosphopeptides (A1 and A2, Fig. 4a) derived from tyrosines 1158, 1162, and 1163, with no detectable formation of mono- or bis-phosphopeptides. The most likely explanation is that a fraction of kinase molecules were basally bis-phosphorylated in this region prior to the addition of $[\gamma-^{32}\text{P}]$ATP. In fact, as assessed by $^{31}$P NMR, the purified kinase possesses 0.35 mol of phosphate/mol of enzyme. This population of kinase molecules would thus undergo rapid bis-phosphorylation. Mono- (C1) and bis- (B2 and B3) phosphopeptides then begin to appear as the previously non-phosphorylated population of kinase molecules undergo a slower rate of autophosphorylation (Fig. 4, b and c). This suggests that bis-phosphorylation may "prime" the kinase to be more active toward phosphorylating itself in trans in a manner analogous to the activation of exogenous substrate phosphorylation by autophosphorylation of the $\beta$-subunit (Rosen et al., 1983; Yu and Czech, 1984). Autophosphorylation of tyrosines 1326 and 1334 is only detectable by 10 min (Fig. 4b), suggesting that autophosphorylation of the soluble kinase is initiated at tyrosines 1158, 1162, and 1163. This would be consistent with our data concerning the time course of phosphorylation of the human placental insulin receptor in vitro (Tavari and Denton, 1988). We have not carried out detailed investigations of the time course of phosphorylation of the 38- or 43-kDa kinases isolated from infected SF9 cells.

As judged by phosphoamino acid analysis, phosphorylation of serine consistently lags behind that of tyrosine (Fig. 5). This is also demonstrated by the slower appearance of peptide S1 compared with the phosphotyrosine-containing peptides (Fig. 4, c versus a). Similarly, we have also found that serine (and threonine) phosphorylation of the native insulin receptor

$^{1}$ B. A. Levine and L. Ellis, unpublished observations.

![Fig. 4. Analysis of the time course of phosphorylation of the 48-kDa soluble tyrosine kinase by two-dimensional phosphopeptide mapping. MonoQ fractions containing predominantly the 48-kDa species were incubated with [\gamma-$^{32}$P]ATP for 5 (panel a), 10 (panel b), or 30 (panel c) min, as described under "Materials and Methods." The kinase was isolated by SDS-PAGE, digested with TPCK-trypsin, and subjected to two-dimensional thin layer chromatography as described under "Materials and Methods." In panel a, the only major peptides to the left of the origin are A1 and A2. Peptides B1 and B3 are only clearly visible in panels b and c, where peptide C1 is apparently obscured by peptide S1. Peptide B2 is only apparent in panel c.](image1)

![Fig. 5. Phosphoamino acid analysis of the time course of phosphorylation of the 48-kDa soluble tyrosine kinase. MonoQ fractions containing predominantly the 48-kDa kinase species were incubated as described under "Materials and Methods." Phosphorylation in the presence of [\gamma-$^{32}$P]ATP was terminated at 5 (lanes 1 and 4), 10 (lanes 2 and 3), or 30 (lanes 3 and 6) min with sample buffer. The 48-kDa kinase was isolated by SDS-PAGE and processed for phosphoamino acid analysis as described under "Materials and Methods." Samples in lanes 1–3 and 4–6 are from parallel incubations using two separate kinase preparations.](image2)

![Fig. 6. Phosphoamino acid analysis of the time course of phosphorylation of the tryptic 38-kDa soluble tyrosine kinase. The tryptic 38-kDa soluble tyrosine kinase was incubated with [\gamma-$^{32}$P]ATP for 2 (lane 1), 6 (lane 2), 12 (lane 3), 18 (lane 4), and 22 (lane 5) min, and phosphoamino acid analysis of the $^{32}$P-labeled soluble kinase was performed as described under "Materials and Methods."](image3)
precipitation as an immune complex with anti-\(\gamma\)-subunit antibodies in "Materials and Methods." This kinase was then incubated with \([\gamma-32P]\)ATP for 2 (panel a), 6 (panel b), 12 (panel c), and 22 (panel d) min, and the \(32^P\)-labeled kinase was isolated by SDS-PAGE for complete tryptic digestion and two-dimensional phosphopeptide mapping, as described under "Materials and Methods." Only peptide C1 is visible in panel a. Peptide B3 becomes apparent in panel b and is clearly visible in panel c along with small amounts of peptides B2, A1, and A2. Peptides A1 and A2 are the only major phosphopeptide species seen in panel d. The material migrating towards the top of panel d probably represents partially digested fragments of kinase.

\(\beta\)-subunit lags behind that of tyrosine in intact \([\beta^{32P}]\)labeled cells transfected with the human insulin receptor cDNA. It is possible, therefore, that insulin-stimulated serine (and threonine) phosphorylation of the insulin receptor is dependent on prior autophosphorylation of the \(\beta\)-subunit. The soluble insulin receptor tyrosine kinase now provides a new experimental system by which to study this phenomenon in more detail.

In our previous studies, phosphoserine was not observed upon incubation of the soluble kinase in vitro with \([\gamma-32P]\)ATP, following either an analytical scale purification of the enzyme by MonoQ chromatography (Cobb et al., 1989) or precipitation as an immune complex with anti-\(\beta\)-subunit antibodies (Ellis et al., 1988). In contrast, the kinase isolated from intact Sf9 cells labeled with \([\beta^{32P}]\)P, was found to contain considerable amounts of phosphoserine, although the phosphorylation sites were not mapped (Ellis et al., 1988). Thus, the scale up of the purification (from a T25 culture flask to 800 ml of cells grown in suspension) and the use of a single preparative MonoQ FPLC anion exchange column, allows the copurification of a serine kinase with the soluble tyrosine kinase.

It is likely that the serine kinase(s) responsible for phosphorylating the insulin receptor soluble tyrosine kinase is/are endogenous to the insect cells rather than an aberrant activity of the tyrosine kinase toward serine residues. Similar serine kinase activities also copurify with the intact insulin receptor during wheat germ lectin-Sepharose chromatography of detergent extracts of human placenta (Ballotti et al., 1986; Smith et al., 1988; Lewis et al., 1990), cells transfected with the human insulin receptor cDNA or rat liver microsomes.

As two-dimensional phosphopeptide mapping demonstrates, at least 6 tyrosines and 2 serines are phosphorylated on the 48- and 43-kDa soluble kinases. We next attempted both to simplify this complex situation, as well as mimic the formation of the 38-kDa kinase, by limited tryptic proteolysis of the 48-kDa kinase. Following proteolysis, the trypsin was removed by passage over a soybean trypsin inhibitor column, and the kinase was repurified by MonoQ FPLC. Analysis by SDS-PAGE reveals the presence of only a single Coomassie Blue-stained band of 38 kDa, which undergoes autophosphorylation and phosphorylates synthetic peptide substrates on tyrosine residues. Phosphorylation of the tryptic 38-kDa kinase is predominantly on tyrosine, with only a trace of phosphoserine, although the phosphorylation of serine again appears to lag behind that of tyrosine (Fig. 6). The level of serine phosphorylation is similar to that found in the 38-kDa kinase isolated from insect Sf9 cells infected with a recombinant Baculovirus (see Fig. 2b, lanes 7–9).

Two-dimensional phosphopeptide mapping (Fig. 7) reveals that autophosphorylation of the tryptic 38-kDa kinase initiates at tyrosines 1158, 1162, and 1163, with the very rapid appearance of peptide C1 (monophosphorylated, Fig. 7a). This was followed by the appearance of peptides B2 and B3 (bis-phosphorylated, Fig. 7, b and c) and finally by peptides A1 and A2 (tris-phosphorylated, Fig. 7d). Indeed, after 22 min of incubation, all peptides from this domain are tris-phosphorylated (A1 and A2, Fig. 7d). This time course thus differs from that of the 48-kDa kinase where there is a very rapid initial appearance of tris-phosphopeptides A1 and A2 followed by a slower rise in mono- and bis-phosphopeptides (see Fig. 4). The difference is most likely to be attributable to the absence of tyrosine-associated phosphate on the purified tryptic 38-kDa kinase. As the 48-kDa kinase undergoes trypsin treatment at 25 °C, and two further purification steps to generate the tryptic 38-kDa kinase, the near complete dephosphorylation of the tryptic 38-kDa kinase could occur during these extra manipulations. Indeed, although we have been unable to obtain sufficient tryptic 38-kDa kinase to confirm this by \(^{31}P\) NMR, the enzyme, prior to incubation with ATP, exhibited no detectable reactivity with anti-phosphotyrosine antibodies in a Western blot analysis.

No phosphorylation of tyrosines 1328 and 1334 (peptide B1) is detectable on the tryptic 38-kDa kinase (Fig. 7), consistent with the data shown in Fig. 3a for the 38-kDa kinase isolated from cultured insect Sf9 cells. Direct protein microsequencing\(^6\) and the lack of reactivity toward an antibody specific for a carboxyl-terminal epitope of the enzyme,\(^7\)

\(^{6}\) T. Issad, J. M. Tavaran, and R. M. Denton, manuscript submitted.

\(^{7}\) B. A. Levine and L. Ellis, unpublished observations.

\(^{8}\) J. M. Tavaran and L. Ellis, unpublished observations.

\(^{9}\) S. Hubbard, W. A. Hendrickson, and L. Ellis, unpublished observations.
have confirmed that the carboxyl terminus of the tryptic 38-kDa kinase is truncated at a position amino-terminal to tyrosines 1328 and 1334. Peptide A1, which we presume to correspond to an amino-terminal autophosphorylation site(s), is also absent, suggesting that some truncation also occurs at the amino terminus.

The fact that the tryptic 38-kDa kinase can proceed to almost complete phosphorylation at tyrosines 1158, 1162, and 1163 (i.e. the exclusive appearance of tris-phosphopeptides A1 and A2 in Fig. 7d) may be indicative of the lack of any phosphatase activity in the final purified product. In all other receptor preparations thus far examined, particularly in intact cells, predominantly bis-phosphorylation of these tyrosines is observed at equilibrium, where there is a delicate balance between kinase and phosphatase activities (Tavaré et al., 1988; Tornqvist et al., 1988; White et al., 1988).

In conclusion, the sites of autophosphorylation of the 48-kDa soluble insulin receptor tyrosine kinase are entirely representative of those found in the native heterotetrameric insulin receptor β-subunit. Limited tryptic proteolysis of the 48-kDa kinase results in the generation of an active core enzyme that autophosphorylates almost exclusively on tyrosines 1158, 1162, and 1163. This autophosphorylation occurs in a cascade mechanism similar to that observed for the intact heterotetrameric insulin receptor. This molecule thus provides an attractive starting material for carrying out further biochemical and biophysical studies on the enzymology of the insulin receptor protein-tyrosine kinase.

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REFERENCES