Differential effect of pp120 on insulin endocytosis by two variant insulin receptor isoforms

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Li Calzi, Sergio, Curtis V. Choice, and Sonia M. Najjar. Differential effect of pp120 on insulin endocytosis by two variant insulin receptor isoforms. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E801–E808, 1997.—The insulin receptor is expressed as two variably spliced isoforms that differ by the absence (isoform A) or presence (isoform B) of a 12-amino acid sequence encoded by exon 11 at the carboxy terminus of the α-subunit. Coexpression of the A isoform and pp120, a substrate of the insulin receptor tyrosine kinase, in NIH 3T3 fibroblasts increased receptor-mediated insulin endocytosis and degradation by two- to threefold compared with cells expressing receptors alone. Because B is the predominant isoform in the liver and binds insulin with lower affinity than A, we have examined the effect of pp120 on receptor B-mediated endocytosis. In contrast to isoform A, the effect of pp120 on isoform B-mediated insulin internalization and degradation in stably transfected NIH 3T3 cells was minimal.

THE INSULIN RECEPTOR is a cell surface glycoprotein of a heterotetrameric structure that consists of two α- and two β-subunits. The extracellular α-subunit contains the insulin-binding domain, and the transmembrane β-subunit contains the tyrosine kinase and the phosphorylation sites. Insulin binding to its receptor activates the receptor’s tyrosine kinase to phosphorylate the receptor and other endogenous substrates. Phosphorylation of substrates mediate the multiple effects of insulin on the cell (25). pp120 is one of the insulin receptor substrates in the liver (10, 18, 20). It is a plasma membrane glycoprotein with a molecular weight (Mw) of ~120,000 that contains phosphorylation sites in its intracellular domain. Site-directed mutagenesis studies in NIH 3T3 mouse skin fibroblasts indicated that pp120 is basally phosphorylated on Ser503 in the absence of insulin and that this phosphorylation is required for insulin-induced pp120 phosphorylation by the insulin receptor tyrosine kinase on Tyr488 residue (16).

An important mechanism to regulate plasma insulin levels is rapid endocytosis of the ligand-receptor complex in response to insulin binding (5). After receptor-mediated endocytosis, insulin is degraded in the lysosomes (27). Insulin degradation is the major mechanism for insulin clearance from the blood, and it mainly occurs in the liver under physiological conditions. Receptor autophosphorylation in response to insulin is involved in this internalization process (1, 7). However, the role of insulin receptor substrates in endocytosis is less defined. Phosphorylation of insulin receptor substrate-1 and activation of phosphatidylinositol 3′-kinase did not appear to regulate insulin-receptor endocytosis in transfected Chinese hamster ovary (CHO) cells (3). In contrast, inhibiting pp120 expression in H4-II-E hepatoma cells by antisense mRNA transfection was associated with a decrease in the internalization and degradation rates of the insulin-receptor complex (4).

The insulin receptor is expressed as two alternatively spliced variants that differ by the absence (isoform A) or presence (isoform B) of a 12-amino acid (aa) sequence encoded by exon 11 at the carboxy (COOH) terminus of the α-subunit. Distribution of the two isoforms is tissue specific, with predominance of isoform B in the liver (21, 22). Although most reports agree that isoform A exhibits higher insulin affinity in cell-free (8, 14) and in intact cell (14, 28) systems, differences in the rate of internalization and in tyrosine kinase activity are less well substantiated. In fact, the observed differences in the rate of endocytosis and in the receptor’s autophosphorylation in transfected Rat-1 (8, 24) and CHO (29) cells were eliminated once these functional measures were corrected for the receptor’s occupancy (12, 29).

The molecular mechanism for the differences in insulin affinity by the inclusion or exclusion of the 12-aa tail in the COOH terminus of the α-subunit of the receptor is not well understood. It has been surmised that changes in the length of the α-subunit induce conformational alterations that modify the receptor’s binding to insulin and its association with other proteins that may play a role in receptor endocytosis (24). Because we have previously reported that coexpression of pp120 and insulin receptors A in NIH 3T3 cells increased insulin-induced receptor endocytosis and degradation rates and that this increase may in part be mediated by the phosphorylation state of pp120 (4), we addressed in this report whether pp120 similarly regulates receptors B-mediated insulin internalization and degradation in transfected NIH 3T3 cells and whether this correlates with a differential kinase activity of the two receptors toward pp120.

MATERIALS AND METHODS

Materials. Lipofectamine Reagent, G418 (geneticin), and protein A-agarose were purchased from Gibco-BRL (Gaithersburg, MD). Hygromycin B was purchased from CalBiochem (La Jolla, CA). 125I-labeled insulin (2,000 Ci/mmol, radioimmunoassay grade), the sheep horseradish peroxidase-labeled anti-rabbit antibody, and the enhanced chemiluminescence (ECL) reagents were purchased from Amersham Life Science (Arlington Heights, IL) and γ-[32P]ATP (6,000 Ci/ mmol) from Du Pont-NEN. Protease inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN). All reagents for polyclarialimide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA). Wheat germ agglutinin (WGA) was purchased from E-Y Laboratories (San Mateo, CA). Human insulin was purchased from Lilly (Indianapolis, IN) and insulin-free bovine serum albumin from Intergen (Des Plaines, IL). Antibodies used in these
studies were previously described (16). Briefly, the monoclonal antibody used to immunoprecipitate pp120 (α-HA4, an identical protein to pp120) was purified from ascites fluid from HA4 c19 cells purchased from the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City, IA). The polyclonal antibody used to immunoblot pp120 (α-295) was raised in rabbit against a peptide (aa 51–64) in the extracellular domain of rat liver pp120. The polyclonal antibody used to immunoblot insulin receptors (Ab-50) was raised against the COOH terminus of the receptor. Monoclonal anti-phosphotyrosine (α-pTyr) antibody and fetal calf serum were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell culture. NIH 3T3 mouse skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Biofluids, Rockville, MD) containing 10% fetal calf serum and 2 mM glutamine (Biofluids). Cells expressing insulin receptors alone and coexpressing human insulin receptors A (hIR-A) and glutamine (Biofluids). Cells expressing insulin receptors alone were grown to 80% confluence and were treated with at least two independent clones for each construct derived from the same transfection.

Quantitation of proteins. Quantitation of the amount of proteins reflected on autoradiograms was achieved by analysis on the Image NIH v1.59 Macintosh software program.

Ligand binding and internalization. Confluent cell monolayers were maintained in six-well plates in triplicate and allowed to grow to ~80% confluence. Cells were incubated overnight at 4°C in binding buffer [100 mM HEPES (pH 7.4), 120 mM NaCl, 1.2 mM MgSO4, 1 mM EDTA, 15 mM CH3COONa, 10 mM glucose, and 1% bovine serum albumin (BSA)] containing 20 pM [50,000 counts·min⁻¹·(cpm)·µl⁻¹] 125I-insulin and were then incubated with prewarmed binding buffer at 37°C for 0–90 min after removal of unbound ligand with ice-cold phosphate-buffered saline (PBS, pH 7.4) (4). At the end of each incubation period, cells were washed three times with ice-cold PBS (pH 7.4) and incubated in 1 ml of 0.1% BSA-supplemented PBS (pH 3.5) for 10 min. The acid wash was then collected to count surface-bound radioactivity that corresponds to noninternalized ligand. Cells were then washed three times with ice-cold PBS (pH 7.4), solubilized in 1 ml ice-cold 0.4 N NaOH-0.1% BSA for 30 min, and collected to count cell-associated radioactivity that corresponds to internalized ligand. Specifically bound ligand was calculated as the sum of surface-bound plus cell-associated ligand. Internalized insulin was calculated as percent cell-associated per specifically bound ligand. Experiments were performed in triplicate and repeated three times on at least two different clones of each cell type. In some experiments, the endocytosis inhibitor phenylarsine oxide (PAO; Sigma Chemical) was added at a final concentration of 250 µM in the binding and incubation buffer (13).

To measure the rate constant (min⁻¹) of insulin internalization, internalization data at earlier time points were fitted to A = Ae⁻kt, where A is a constant, k is the rate constant, and t is time per min by use of a Marquardt-Levenberg nonlinear least squares algorithm (19). The MathSoft AXUM 4.1 Program for personal computer was employed for this analysis.

Insulin degradation. After solubilization of cells in 1 ml ice-cold 0.4 N NaOH-0.1% BSA, as described in Ligand binding and internalization, a 400-µl aliquot was collected to count total cell-associated radioactivity, and another 400-µl aliquot was subjected to precipitation in 10% trichloroacetic acid (TCA)-0.1% BSA for 30 min at 4°C. After centrifugation of the TCA pellet (12,000 g for 10 min at 4°C), the supernatant was collected in addition to the first wash of the TCA pellet and counted as a measure for TCA-soluble radioactivity. Intracellularly degraded insulin was calculated as percent TCA soluble per internalized insulin (see Ligand binding and internalization). Experiments were performed in triplicate and repeated four times on at least two different clones of each cell type.

Insulin retroendocytosis. Retroendocytosis of insulin was measured as described in Ref. 30. Briefly, cells were incubated in incubation buffer at 37°C for 20 min after removal of unbound 125I-insulin. Surface-bound ligand was removed with acid, and cells were reincubated in binding buffer for 0–120 min. At each time point, the medium was collected for direct counting and TCA precipitation. Cells were washed in PBS (pH 7.4) and lysed in NaOH to determine cell-associated radioactivity as before. Retroendocytosed insulin was calculated as percent TCA precipitable per released ligand. Degraded insulin was calculated as percent TCA soluble per released ligand. Experiments were performed in triplicate and repeated six times on at least two different clones of each cell type.
Statistical analysis. Curves were compared by a multivariate analysis of variance, and individual points were compared by paired t-tests. P values of <0.05 were considered statistically significant.

RESULTS

Insulin receptor isoform B phosphorylates pp120. To compare the ability of both receptor isoforms to phosphorylate pp120, we examined phosphorylation of equal amounts of partially purified pp120 (from NIH 3T3 cells overexpressing wild-type full length pp120) by partially purified receptor A and B isoforms (from NIH 3T3 cells individually overexpressing either human receptor isoform). Because insulin induced equal auto-phosphorylation of human insulin receptors A (hIR-A) or B (hIR-B) at 10^{-7} M (12), we allowed insulin at this high concentration to bind to the receptors for 1 h at room temperature before phosphorylating in the presence of \gamma-[32P]ATP. Two-thirds of the phosphorylation product were immunoprecipitated with a monoclonal antibody directed against pp120 (Fig. 1A), and the rest was immunoprecipitated with an \alpha-pTyr monoclonal antibody (Fig. 1C) before analysis by SDS-PAGE. Corrected for the higher receptor’s number in the clone transfected with hIR-B, as evidenced by the twofold higher level of immunoblotted hIR-B than hIR-A (Fig. 1D, lanes 8 vs. 6), insulin equally stimulated autophosphorylation of hIR-A (Fig. 1C, lanes 5 vs. 6) and hIR-B (Fig. 1C, lanes 7 vs. 8). In addition, ligand-stimulated phosphorylation of an \sim 120,000-M_r band corresponding to recombinant pp120 was mediated by both receptor isoforms by approximately twofold relative to basal (Fig. 1A, lanes 1 vs. 2 and lanes 3 vs. 4), with equal amounts of immunoprecipitable pp120 applied to the phosphorylation assay in the absence and presence of insulin (Fig. 1B, lanes 2 vs. 1 and lanes 4 vs. 3). Identical results were obtained with another clone derived from the same transfection for each construct. The antigen-pp120 antibody complex from cell extracts of NIH 3T3 cells transfected with neomycin-resistant cDNA alone revealed no phosphorylation by lectin-purified receptors (data not shown), supporting our hypothesis that the \sim 120,000-M_r band is specific to the pp120 protein.

As we have previously reported (16), insulin stimulated the phosphorylation of an additional species with M_r of \sim 95,000 (band y) that was immunoprecipitated by monoclonal antibody directed against pp120 (Fig. 1A, odd-numbered lanes). Insulin-stimulated phosphorylation of this species in cells expressing hIR-B is less marked than in cells expressing hIR-A (Fig. 1A, lanes 3 vs. 1) despite higher hIR-B autophosphorylation (Fig. 1C, lanes 7 vs. 5), suggesting that this band is not the...
Insulin internalization (endocytosis). Cell-associated $^{125}\text{I}$-insulin was measured in NIH 3T3 cells expressing comparable amounts of insulin receptors per cell in each hIR-A and hIR-B clone, with or without pp120. To overcome differences in insulin affinity of hIR-A and hIR-B, insulin binding at 4°C was carried out overnight. The most significant, albeit modest, effect of pp120 on cell-associated radioactivity in cells coexpressing hIR-B was observed during early incubation at 37°C (Fig. 2B, inset, 0–8 min). This effect was essentially abolished at later times. At 8 min of incubation at 37°C, 22 ± 3% of bound hormone was detected in cells expressing hIR-B alone (Fig. 2B, inset, hIR-B [14]). As the inset of Fig. 2B reveals, this ratio was marginally raised to 26 ± 1 and 27 ± 1% when pp120 was coexpressed [hIR-B/pp120 (18) and hIR-B/pp120 (7), respectively]. After the same incubation time at 37°C, 12 ± 1% of bound insulin was detected in cells expressing hIR-A alone (Fig. 2A, inset, hIR-A/pp120 [3006]), and this ratio was markedly increased to 42 ± 2% ($P < 0.05$) when pp120 was coexpressed (Fig. 2A, inset, hIR-A/pp120 [10]). Thus the upregulatory effect of pp120 on cell-associated radioactivity appeared to be more specific for the insulin receptor A isoform. As Fig. 2B reveals, the half-time ($t_{1/2}$) of cell-associated radioactivity in cells expressing hIR-B alone [hIR-B (14)] was 9 min and was reduced to 5 min when pp120 was coexpressed [hIR-B/pp120 (18) and hIR-B/pp120 (7)]. The $t_{1/2}$ of cell-associated radioactivity was 20 min in cells expressing hIR-A alone (Fig. 2A, hIR-A [3006]) and was decreased to ~1 min when pp120 was coexpressed (Fig. 2A, inset, hIR-A/pp120 [10]). This suggests that pp120 increased the rate of insulin association with cells coexpressing insulin receptors A to a much more significant level (~1 vs. 20 min) than that with cells coexpressing insulin receptors B (5 vs. 9 min). These results were reproduced in at least one other clone from each transfection. For simplicity, only two clones (18 and 7) of cells coexpressing pp120 and hIR-B were shown in Fig. 2. These two clones were chosen for their different pp120 expression. Despite the tenfold lower pp120 expression in clone 18 than in clone 7 (data not shown), the effect of pp120 on cell-associated insulin reached saturation in transfected cells (4). pp120 levels in transfected cells were significantly higher than endogenous pp120 levels in liver cells.

To test whether the observed effect of pp120 on cell-associated radioactivity in cells coexpressing hIR-A was a reflection of increased receptor affinity for insulin or indicative of increased insulin endocytosis, we measured cell-associated radioactivity in hIR-A-expressing cells in the absence or presence of PAO, an endocytosis inhibitor. As Fig. 3 reveals, PAO markedly decreased the amount of cell-associated radioactivity in cells expressing hIR-A alone (Fig. 3A) or coexpressing pp120 (Fig. 3B), suggesting that pp120 upregulates cell-
associated radioactivity by increasing insulin endocytosis in cells expressing hIR-A.

To better assess the difference in the effect of pp120 on insulin internalization in cells coexpressing either hIR-A or hIR-B, the internalization rate constant, \( k \), was estimated by fitting internalization data at earlier time points in a first-order equation using a Marquardt-Levenberg nonlinear least squares algorithm (19). Internalization at lower incubation time was examined in this analysis to avoid potential interference by recycled insulin after prolonged incubation at 37°C. In concordance with our previous observations (4), \( k \) (min\(^{-1}\)) was two- to threefold higher in cells coexpressing hIR-A and pp120 than in cells expressing hIR-A alone [Fig. 4A, 45.0 ± 4.7 in hIR-A/pp120 (10) vs. 21.6 ± 1.5 in hIR-A (3006), \( P < 0.05 \)]. In marked contrast, \( k \) (min\(^{-1}\)) was virtually identical in cells expressing either hIR-B alone or coexpressing hIR-B and pp120 [Fig. 4B, 57.3 ± 3.5 in hIR-B (14) vs. 45.6 ± 2.3 in hIR-B/pp120 (18); \( P > 0.05 \)]. Thus it appears that pp120-induced increase in the rate of insulin endocytosis is specific for insulin receptors A-mediated ligand endocytosis.

**Insulin degradation.** After an overnight insulin binding at 4°C, insulin degradation was measured by incubating cells at 37°C for 0–90 min. Surface-bound radioactivity was removed by an acid wash and collected to count noninternalized ligand. Cell-associated radioactivity corresponding to internalized ligand was subjected to TCA precipitation. Failure of internalized insulin to precipitate with TCA was used as a measure for intracellular lysosomal insulin degradation. Because insulin degradation normally begins after internalization, we compared the ratio of degraded per internalized insulin after 20 min of incubation at 37°C before potential ligand recycling occurred. As shown in Fig. 5B, 28 ± 2% of internalized insulin was degraded in cells expressing hIR-B alone [hIR-B (14)]. When pp120 was coexpressed [hIR-B/pp120 (7)], this ratio was modestly increased by 1.3-fold to 37 ± 2% (\( P < 0.05 \)). In contrast, the effect of pp120 expression on insulin degradation was more marked in cells coexpressing hIR-A. Whereas 25 ± 2% of internalized insulin was degraded in cells expressing hIR-A alone [Fig. 5A, hIR-A (3006)], 62 ± 2% of internalized insulin was degraded in cells coexpressing hIR-A and pp120 [Fig. 5A, hIR-A/pp120 (10)], indicating a 2.5-fold increase in insulin degradation in the presence of pp120 in cells expressing insulin receptors A. Thus pp120 exerted a specific effect on insulin degradation after ligand internalization via insulin receptors A. This differential effect on insulin degradation paralleled its differential effect on insulin internalization.

**Insulin recycling (retroendocytosis).** Because the effect of pp120 on insulin endocytosis and degradation, albeit modest in cells expressing hIR-B, appeared to be more significant at early time points and because it
decreased as postinternalization time increased (Figs. 2 and 5), we proposed that the effect on later events, such as ligand recycling, is minimal. To evaluate this hypothesis, we measured the effect of pp120 on insulin recycling by incubating cells for 20 min at 37°C to allow sufficient internalization (30). After removal of noninternalized surface-bound insulin in acid, cells were reincubated in incubation buffer at 37°C for 0–120 min. At the end of each time point, released radioactivity in the medium was subjected to TCA precipitation. The relative amount of TCA-soluble radioactivity was determined to measure lysosomally degraded ligand, whereas the TCA-precipitable radioactivity was determined to measure the relative amount of ligand that would recycle back into the cell during prolonged reincubation at 37°C. The relative amounts of TCA-soluble and TCA-precipitable radioactivity reached a plateau at 30–120 min in all cell lines examined (data not shown); hence, the relative amounts of degraded and retroendocytosed insulin after 120 min of incubation at 37°C were compared (Fig. 6). In cells expressing hIR-A alone [hIR-A (3006)], 11.25 ± 4.73% of released insulin was retroendocytosed after 120 min of incubation at 37°C. The relative amount of retroendocytosed ligand was virtually unchanged (8.21 ± 0.54%, P > 0.05) when pp120 was coexpressed [Fig. 6A, hIR-A/pp120 (10)]. After the same incubation time at 37°C, identical relative amounts of released ligand retroendocytosed in cells expressing either hIR-B alone or coexpressing hIR-B and pp120 were observed (Fig. 6B, 6.79 ± 2.61% in hIR-B (14) vs. 8.30 ± 1.38% in hIR-B/pp120 (7), P > 0.05). Thus pp120 did not significantly alter insulin retroendocytosis in cells cotransfected with either receptor isoform.

**DISCUSSION**

After insulin binding to its receptor, the ligand-receptor complex is rapidly internalized (11). Under...
normal physiological conditions, the internalized receptor is recycled to the plasma membrane, whereas insulin is targeted for lysosomal degradation (30). Receptor-mediated insulin endocytosis is the main mechanism for insulin clearance from the circulation. Regulation of this process has profound pathophysiological consequences in fuel metabolism. For example, chronically impaired ability to clear insulin from the circulation, as seen in certain liver diseases, causes hyperinsulinemia with attendant peripheral insulin resistance.

The insulin receptor gene is expressed as two alternatively spliced isoforms that differ by the absence (hIR-A) or presence (hIR-B) of a 12-aa segment encoded by exon 11 at the COOH terminus of the α-subunit of the receptor. Expression of the two isoforms is regulated in a tissue-specific manner (21). Adult hepatocytes express the highest relative levels of the insulin receptor B isoform in humans (21). The two isoforms differ in their insulin-binding affinity, with a higher affinity for insulin receptors A. It has been suggested that the different binding affinity of the two isoforms may represent a mechanism whereby hepatic insulin receptors are rendered less sensitive to insulin-induced receptor downregulation. This mechanism could protect the liver against physiologically elevated concentrations of insulin in the portal blood, which are two- to threefold higher than insulin concentrations in the peripheral circulation (14).

It is interesting that pp120 is mainly expressed in the liver, a major site of insulin clearance from portal circulation. In this report, we presented evidence that pp120 upregulated insulin endocytosis and degradation when mediated by the high-affinity insulin receptors A but not by insulin receptors B. The differential effect on insulin internalization and degradation was not associated with altered insulin recycling. These data are consistent with a physiological model in which pp120 promotes insulin internalization by insulin receptors A to preferentially target insulin to intracellular lysosomes, where it undergoes degradation. Thus pp120 may be part of a complex of proteins that differentially regulate internalization of the two receptor isoforms and ligand degradation in the liver. By increasing insulin-induced receptor internalization, pp120 may regulate the relative distribution of the two receptor isoforms in the hepatocyte. For instance, it is reasonable to predict, on the basis of our observations, that in a state of high plasma insulin levels, pp120 would increase insulin removal via receptors A, preventing downregulation of the high-affinity A receptors. Thus regulation of the relative sensitivity of the two receptor isoforms by a pp120-dependent pathway may have potential implications in the mechanism of insulin resistance.

It has been reported that dexamethasone, a synthetic glucocorticoid, decreases the relative level of the high-affinity insulin receptors A in HepG2, a human hepatoma cell line (9, 17). In light of the fact that the expression of pp120 increases in livers of rats treated with dexamethasone (20), we would expect on the basis of our findings that pp120 increases sensitivity of the high-affinity insulin receptors A to insulin to offset the decreased insulin clearance that results from reduced insulin receptors A in the hepatocyte.

The molecular mechanism for ligand endocytosis is not clear. It has been suggested that internalization of the insulin-receptor complex is a two-step process. The first step involves redistribution of the receptors from the microvilli to the nonvillous membrane of the cell, whereas the second step consists of anchoring the receptors in clathrin-coated pits (3). Requirement for receptor autophosphorylation for these two steps has been controversial (3, 23). In this report, we observed that pp120 differentially increased insulin receptor A-mediated internalization despite being equally phosphorylated by both receptor isoforms. This suggests that the pp120-induced increase in insulin endocytosis does not depend on its phosphorylation state. However, expression of a phosphorylation-defective splice variant of pp120 (15) and of site-directed mutants, in which phosphorylation sites (Tyr488 and Ser503) were replaced with nonphosphorylatable amino acids, abolished the effect of pp120 on insulin-insulin receptor A complex endocytosis (4). Thus pp120 phosphorylation appears to be necessary but not sufficient to mediate the effect of pp120 on insulin endocytosis. It remains possible that pp120 exerts its effect by targeting the receptor into clathrin-coated pits. In fact, the amino acid sequence in proximity to Tyr488 (Tyr-Ser-Val-Leu) shares high homology with tyrosine-centered motifs known to play a significant role in interacting with adaptin molecules that anchor proteins in clathrin-coated pits (6). Further studies are required to elucidate the exact mechanism for the upregulatory effect of pp120 on receptors A-mediated insulin internalization and degradation.

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REFERENCES
5. Gorden, P., J.-L. Carpentier, P. Freychet, A. LeCam, and L. Orci. Intracellular translocation of iodine-125-labeled insulin:


