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Neuronal Insulin Receptors in Y79 Retinoblastoma Cells

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SUMMARY: Immunoblot analysis of Y79 cell membrane proteins indicated that Y79 insulin receptors (InsRs) α subunits had a mass of 115 kDa. Biosynthetic studies revealed a typical transit time for InsR delivery to the Golgi (~2h) and receptor processing. However, neither the proreceptor nor the mature receptor exhibited endoglycosidase H-resistance, consistent with a lack of *N*-linked glycan processing. Insulin stimulated a rapid and transient tyrosine phosphorylation of receptor β subunits (95 kDa) and of IRS-1 in intact Y79 cells, whereas *in vitro* studies with enriched membrane glycoproteins resulted in the autophosphorylation of both InsR (95 kDa) and IGF-1-R (98k Da) β subunits. These studies provide the first biochemical dissection of InsR structure and function in retinoblastoma cells.

The insulin and insulin like growth factor-1 (IGF-1) receptors share considerable similarities both in structure and function (6, 9, 22, 37, 50). Each receptor consists of a tetrameric complex of 2 α and 2 β subunits linked by disulfide bridges. The α subunits are completely extracellular and are responsible for ligand binding (53). The β subunits are transmembrane proteins containing within their intracellular portion, ligand stimulatable tyrosine kinase activity and at least 5 sites for receptor autophosphorylation (47). Postulated substrates for these tyrosine kinases include IRS-1 (45), IRS-2 (2) and the the adaptor molecule SHC (34). These phosphorylated proteins in turn serve as binding sites for proteins containing SH2 domains (27), thereby forming multisubunit signaling complexes that regulate downstream transduction pathways through Ras activation (3)

Size differences between neuronal and peripheral InsRs with neuronal α subunits having a mass of ~115 kDa *vs.* 125 kDa for peripheral InsR α subunits are thought to arise from differential glycosylation (14, 26, 54). These findings imply that InsR subtypes exist between neuronal and peripheral tissues with the differences potentially reflecting alterations in insulin's impact on nervous tissue (11). Roth et al. (40) identified a 120 kDa protein in human brain membranes that may be an IGF-1 receptor. This is consistent with analyses of IGF-1Rs in neuronal cells derived

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ABBREVIATIONS: InsR, insulin receptor; IGF-1-R, insulin-like growth factor-1 receptor; α-pY Ab, antiphosphotyrosine antibody; IRS-1, insulin receptor substrate-1; SH2, src homology region 2; endo H, endoglycosidase H; WGA, wheat germ agglutinin; TPCK trypsin, N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin; MAP kinase, mitogen activated protein kinase.

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from brain which exhibit a mass of 115 kDa compared to 135 kDa for glial cell IGF-1Rs (4, 43). In addition, alternative patterns of exon splicing have been observed for the InsR α subunit (9, 49). One InsR variant (hIR-B) has an extra exon (exon 11) and is ~1.5 kDa larger than the other splice variant, hIR-A. hIR-B is found mainly in the liver and has an approximately two-fold lower affinity for insulin than hIR-A (28).

In several models of diabetes, peripheral InsRs are up-regulated in the kidney (30), adipose tissue (8) and liver (19). This was also found to pertain in the retinae of humans with type 1 diabetes (39) and streptozotocin-treated rats (56). This up-regulation, coupled to a decrease in vascular InsRs (56), may be a determining cause of or distinctive symptom of diabetic retinopathy, which is characterized by retinal microvascular and neural damage, ultimately leading to blindness. The Y79 retinoblastoma cell line represents a pluripotential clonal cell line derived from a tumor of the inner plexiform layer of the retina (23) that retains many neuronal characteristics including the surface expression of InsRs (41). To better understand the structure, function and regulation of InsRs in the central nervous system and more specifically in the human retina, we have studied these receptors in Y79 cells as a model for the neural retina.

MATERIALS and METHODS

Materials

CH-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Pansorbin and endoglycosidase H were from Calbiochem (La Jolla, CA). The ECL reagent was from Amersham (Arlington Hts, IL). [γ-^{32}P]ATP (6,000 Ci/mmol) ^{35}S-methionine and ^{32}P-orthophosphoric acid were from Dupont-New England Nuclear (Boston, MA). Crystalline porcine insulin was the generous gift of Dr. R. Frank, Eli Lilly & Co. (Indianapolis, IN). Recombinant human IGF-1 was kindly provided by Genentech, Inc. (South San Francisco, CA). Alkaline phosphatase-conjugated goat anti-rabbit antibodies were from Promega (Madison, WI) and peroxidase-conjugated goat anti-rabbit antibodies were from Chemicon International, Inc. (Temecula, CA). The preparation of rabbit antiphosphotyrosine IgG (α-pY), α-InsR and α-IGF-1-R antibodies has been described elsewhere (33, 39). Wheat germ agglutinin-Sepharose was purchased from Vector (Burlingame, CA). PMSF, aprotinin, BSA, *N*-acetyl D-glucosamine, and bacitracin were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose paper was from MSI Separations, Inc. (Westboro, MA). 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT) were from BioRad Laboratories (Richmond, CA). All other chemicals were reagent grade.

Gel electrophoresis and autoradiography

Proteins were analyzed by SDS-PAGE according to the method of Laemmli (24). After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue. The dried gels were exposed to Kodak XAR-5 x-ray film for 1-14 days at -80°C using a Dupont Cronex Lightning Plus intensifying screen. For fluorography, gels were impregnated with a solution of 1 M salicylate prior to drying.

WGA-agarose chromatography

For affinity chromatography, two ml of WGA-Sepharose was equilibrated in 50 mM HEPES, pH 7.5 containing 0.1 M NaCl and 0.1% v/v Nonidet P-40. In preparation for chromatography, membranes (1-2 mg) were solubilized in 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM $MgCl_2$, 0.2% BSA, 0.01% STI, 1 mM bacitracin, 1 mM PMSF, 2% v/v Nonidet P-40 by incubation on ice for 10 min and gentle mixing for 5 min. Insoluble material was removed by centrifugation at 15,000 x g for 30 min. The soluble fraction was diluted to 0.2% v/v Nonidet P-40 with 50 mM HEPES, 0.1 M NaCl and added to the WGA-Sepharose beads packed in a column. The flow-through was recycled three times and the gel was sequentially washed with 0.1 M NaCl, 0.5 M NaCl, and finally 0.3 M GlcNAc in 0.5 M NaCl all in 50 mM HEPES, pH 7.5 containing 0.1% v/v Nonidet P-40. The eluate was concentrated to ~1 mg/ml in a Centriprep 30 and frozen as aliquots at -80°C until use.

Immunoblot analysis of receptor α subunits

Insulin receptor α subunits were immunoblotted with site-specific anti-peptide antiserum as previously described (39). Proteins resolved on SDS gels were transferred onto nitrocellulose

which was then quenched for at least 60 min in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% v/v Tween 20) containing 5% nonfat dry milk (17). Filters were then incubated with affinity purified antibody at 1:100 dilution (2 μg/ml) in the same buffer for 18 h at 4°C and developed with the indicated goat anti-rabbit secondary.

Pulse-labeling of cells and Endo H digestion

Cells were pulse-labeled for 15 min with 0.5 mCi ^{35}S methionine, washed in excess methionine containing medium and aliquots were chased at 37°C for the times indicated. At each time point, aliquoted cells were washed with 5 ml methionine fortified medium and solubilized in 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM $MgCl_2$, 2% v/v Nonidet P-40, 1 mM bacitracin, 1 mM PMSF, 1 μg/ml aprotinin and 5 μM leupeptin. InsRs were immunoprecipitated by overnight incubation and the recovered immune precipitates were suspended in 50 μl of 0.1 M sodium citrate pH 5.5 containing 0.1% Nonidet P-40, 0.1% SDS and 1 mM PMSF. 25 μl from each aliquot was incubated (12 h, 37°C) with 5 mU Endo H or with buffer only. Digestions were terminated by addition of SDS sample buffer and boiling for 3 min.

Autophosphorylation of receptors

20 μg of WGA-Sepharose purified membrane protein was incubated in 50 mM HEPES pH 7.4 containing 0.1% v/v Nonidet P-40 and 2 mM $MnCl_2$ in the presence of the indicated ligand for 20 min on ice. The samples were subsequently incubated for 30 min at 23°C with [γ-^{32}P]ATP (2.5 μCi, 25 μM ATP). The reaction was stopped by addition of sample buffer and the samples were boiled for 3 min prior to electrophoresis on 7.5% SDS polyacrylamide gels. The gels were stained, dried and exposed to Kodak XRP x-ray film for 4-24h at -80°C.

Trypsinization of InsRs

16 μg of WGA-agarose enriched Y79 membrane proteins were treated with TPCK-trypsin for 3 min at 23°C. The reaction was stopped by the addition of soybean trypsin inhibitor (0.16% w/v final) and [γ-^{32}P]-ATP (3 mM/15 μCi) and 8 mM $MnCl_2$ were added for 20 min at 23°C. 150 μl of buffer A was added (50 mM HEPES pH 7.4, 10 mM sodium pyrophosphate, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 1% v/v Nonidet P-40, 1 mM PMSF, 100 U/ml aprotinin) and the samples were immunoprecipitated with α-pY and phosphoproteins visualized by SDS-PAGE and autoradiography of the dried gel. WGA-agarose enriched human placental membranes (15; 100 μg) were trypsinized for 1 or 3 min as described. Each sample was divided in two, resolved by non-reducing SDS-PAGE and immunoblotted with α-pY and α-InsR.

Immunoprecipitation of tyrosine phosphorylated proteins

Aliquots of 10^7 serum starved Y79 cells were incubated in phosphate-free modified Eagle's medium containing ^{32}P-orthophosphate for 2 h at 37°C. After washing the cells in phosphate-free medium, they were stimulated with 10^{-7} M insulin and frozen in liquid N_2. Following solubilization in 0.5 ml of buffer A, the extracts were incubated for 15 min at 4°C and insoluble material pelleted in a microfuge (12,000gav/4°C/20 min). The supernatants were kept for immunoprecipitation and 100 μl of 1% SDS in buffer A was added to each pellet, and resuspended. Additional buffer A (0.9ml) was added and the samples were cleared by incubation for 1 h at 4°C with 50 μl of Pansorbin. Antibody was added to the cleared supernatants and after 18 hr at 4°C the immune complexes were recovered with Pansorbin, solubilized in sample buffer and analyzed by SDS-PAGE and autoradiography.

RESULTS and DISCUSSION

Immunoblotting of InsRs. Based on insulin binding assays (41, 55), Y79 cells are known to express functional InsRs, however, the structural properties of these InsRs have not been characterized. Using immunoblot analysis with site-specific antibodies raised against a peptide sequence present in the COOH-terminal of InsR α subunit sequence, the apparent mass of InsRs from Y79 cells was compared to that of InsRs from rat and human liver membranes and human neuroblastoma cell membranes. As shown in Fig. 1, Y79 cell InsR α subunits were ~115 kDa in size having an electrophoretic mobility comparable to that of neuronal InsR α subunits (SK-N-MC cells) but smaller than their peripheral counterparts in liver membranes (115 kDa *vs.* 125 kDa). Of note, we could not detect InsRs in membranes prepared from Rin 5F cells, a rat insulinoma cell line (12). This is presumably due to their low level of expression, perhaps resulting from insulin-induced down regulation.

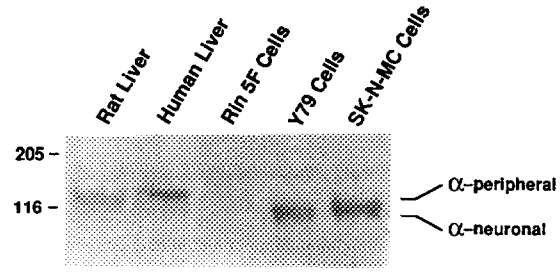

Figure 1. Immunoblot of InsR α subunits.
Membrane proteins (100 μg) from the indicated sources were resolved on a 6.5% SDS gel, transferred to nitrocellulose and immunoblotted with α-InsR Ab. The blot was developed with alkaline phosphatase-conjugated goat anti-rabbit IgG according to standard techniques. Rat insulinoma-Rin5F cells; retinoblastoma-Y79 cells; neuroblastoma-SK-N-MC cells.

Biosynthesis of InsRs. To establish the kinetics of InsR biosynthesis, Y79 cells were pulse-labeled for 15 min with ^{35}S-methionine followed by chase incubation. Following immunoprecipitation with α-InsR IgG, the immune complexes were treated with endo H to assess the delivery of InsRs to the Golgi complex. As shown in Figure 2, the InsRs remained endo H sensitive throughout the entire experiment (4 h) indicating a lack of maturation of their *N*-linked oligosaccharides. This is consistent with differential glycosylation of neuronal InsRs as being

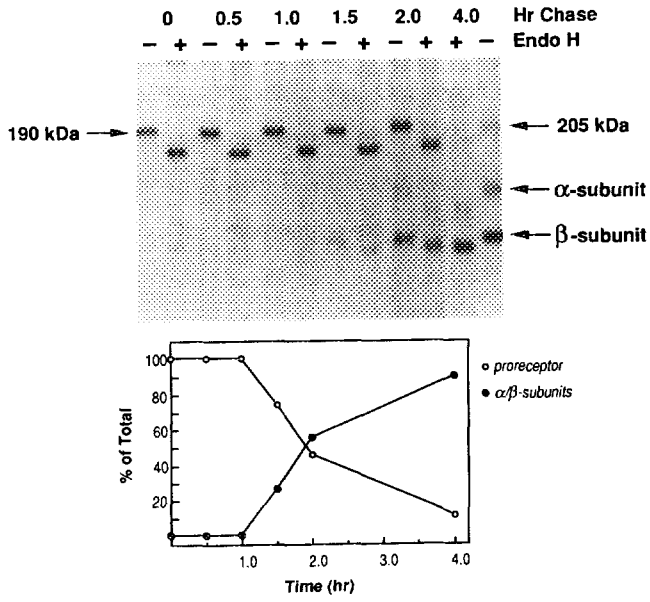

Figure 2. Kinetics of InsR biosynthesis in Y79 retinoblastoma cells.
Y79 cells were pulse-labeled for 15 min with ^{35}S-methionine as described in Methods. Shown is a 7 day exposure salicylate-impregnated fluorogram.
Upper: Fluorogram. *Lower*: Quantification of the net cpm present in proreceptor vs α/β subunits at each time point as determined by densitometric scanning of the x-ray film.

responsible for their smaller molecular mass. A discernible decrease in the electrophoretic mobility of the proreceptor (and β subunit) was observed after 2 h of chase, indicating delivery to the medial/trans Golgi. At this time, 50% of the α/β proreceptor was converted to individual α and β subunits. This suggests that receptor converting enzyme is also localized to the medial-trans Golgi. The ease of studying InsR biosynthesis suggestes it may be a model system for the investigation of proreceptor:receptor processing and receptor regulation; both of which altered in diabetes (7, 30).

Receptor autophosphorylation. As reported for neuroblastoma cell lines (32, 43), and primary neuronal cell cultures (25), we also observed the phosphorylation of two distinct β subunits in *in vitro* phosphorylation experiments (Fig. 3A). A doublet has also been observed in fetal muscle (1) and cell lines other than retinoblastoma cells (29) which has been interpreted as resulting from IGF-1R β subunit phosphorylation. It has been suggested that the mechanism by which insulin stimulates IGF-1R β subunit phosphorylation may be due to the existence of hybrid heterotetramers (29). Figure 3B shows that in Y79 cells, the 95 kDa band is the InsR β subunit and the 98 kDa band is the β subunit of the IGF-1-R; both are phosphorylated in response to insulin in

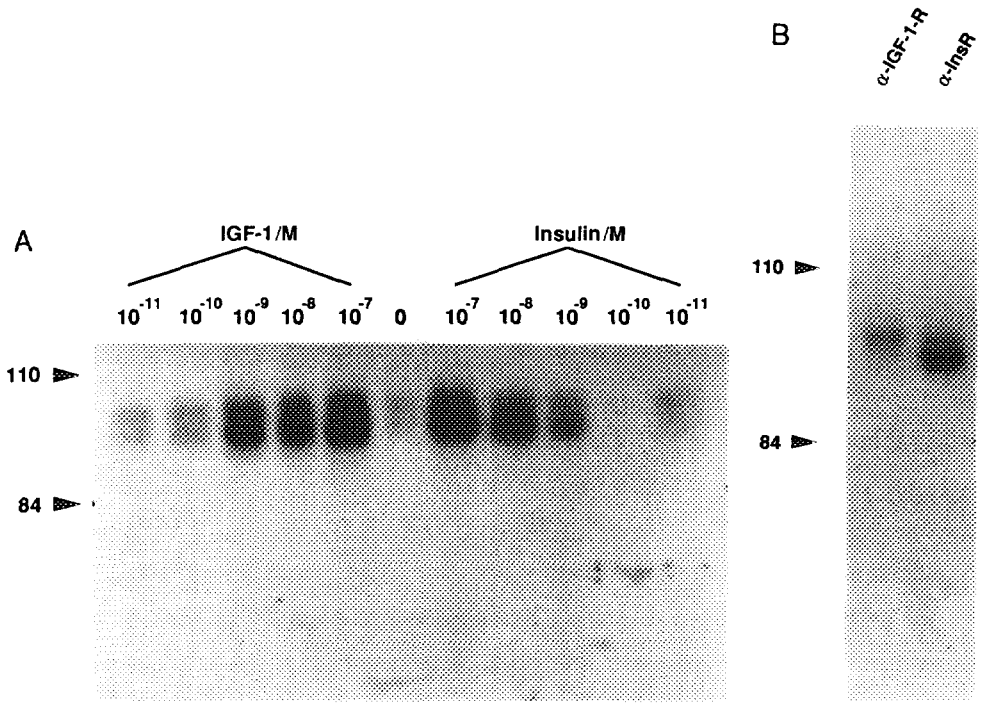

Figure 3. Insulin and IGF-1 induced receptor autophosphorylation.
3A. 10 μg of WGA-agarose enriched membranes from Y79 cells were incubated with 5 mM $MnCl_2$ and the indicated concentration of insulin or IGF-1 for 20 min on ice followed by 30 min at 23°C in the presence of 10 μCi of γ-^{32}P-ATP (2,500Ci/mmol). Tyrosine phosphorylated proteins were immunoprecipitated with α-pY IgG and analyzed by SDS polyacrylamide gel electrophoresis and autoradiography of the dried gel.
3B. Y79 membranes were autophosphorylated by addition of 10^{-7} M insulin and immunoprecipitated with α-pY IgG as described in the legend to Fig 3A. Immunoprecipitated proteins were split in half; one half was immunoprecipitated with α-InsR and the other with α-IGF-1-R and analyzed by SDS-PAGE and autoradiography of the dried gel.

partially purified solubilized membranes, indicating a lack of hybrid heterotetramer formation in these cells.

In response to insulin and IGF-1, there is a rapid, reversible and selective increase in the level of tyrosine phosphorylation of cellular proteins including IRS-1, MAP kinase and nuclear proteins in a number of cell lines (10, 13, 16, 31, 36, 48, 51). Tyrosine kinase substrates in neural cells include IRS-1 and a 70 kDa species (18, 42). As shown in Fig. 4, IRS-1 was rapidly (maximum incorporation in 30 sec) and reversibly phosphorylated in response to insulin. It was present in the non-ionic detergent extract of Y79 cells and absent in the SDS extract of the Nonidet P-40 insoluble pellet. This suggests a lack of tyrosine phosphorylation of proteins associated with the cytoskeleton, as reported for a 175 kDa substrate of the InsR in FRTL5 thyroid cells (5). Consistent with the above *in vitro* evidence for the lack of hybrid heterotetramers in Y79 cells, we did not observe phosphorylation of two β subunits in response to insulin in intact Y79 cells (Fig. 4). These data suggest that phosphorylation of this second β subunit may not be of functional significance (i.e., it does not occur in intact cells). This may be due to the IGF-1R being an *in vitro* substrate of the InsR in WGA-agarose enriched detergent extracts of these cells only.

Trypsin activation of InsRs. Trypsin's insulin mimetic effects in intact tissues (20, 21, 38), despite causing reduced insulin binding (35) and the increased autophosphorylation of trypsinized, partially purified InsRs (46) has suggests that cleavage of the extracellular α subunit results in tyrosine kinase activation of the β subunit, perhaps through removal of the inhibitory influence of the α subumit. Trypsinization of partially purified rat InsRs results in the cleavage at Arg^{576}-Arg^{577}, yielding a 110 kDa α/β heterodimer with constitutively active tyrosine kinase activity (44). Trypsin cleavage of human placental InsRs yielded species ranging from 53 kDa to

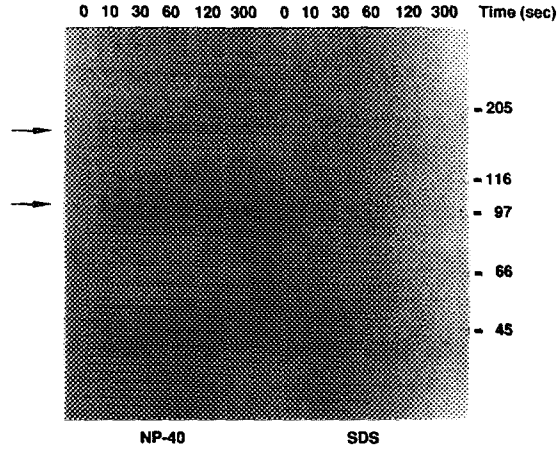

Figure 4. Insulin-induced phosphorylation of IRS-1 in Y79 cells.
Y79 cells were serum-starved for 24h followed by preincubation in phosphate-free medium supplemented with 250 μCi/ml ^{32}Pi. Aliquots of 10^7 cells were treated with insulin (0.1μM) for the indicated times, after which the cells were lysed in detergent-containing buffer containing phosphatase and protease inhibitors. Material which was insoluble in Nonidet P-40 was solubilized in SDS; this consists mainly of cytoskeletal and nuclear proteins. Tyrosyl-phosphorylated proteins were immunoprecipitated from all samples using 7.5 μg of α-pY IgG. The phosphorylated substrates were eluted from the immune complexes by the addition of 17 mM phosphotyrosine and analyzed by SDS polyacrylamide gel electrophoresis and autoradiography of the dried gel.

175 kDa, none of which retained kinase activity (52). As shown in figure 5A, trypsin treatment of membranes from Y79 cells and SK-N-MC cells resulted in the production of four active species of 100 kDa, 85 kDa, 78 kDa and 53 kDa. In a similar experiment, trypsinization of human placental membranes generated a 60 kDa protein which was detectable with α-pY and α-InsR IgGs, indicating it is a constitutively active α/β heterodimer (Figure 5B); a 140 kDa receptor fragment lacking autophosphorylation activity was also generated. Analysis of IGF-1Rs in this manner did not yield any constitutively active species (data not shown). The relationship between the active placental 60 kDa fragment and the active fragments arising from trypsinization of the neuronal InsR remains to be determined.

The functional impact of insulin on the neural retina is at present poorly understood. However, it may regulate the differentiation/neurite outgrowth of subpopulations of neuronal cells in the developing retina, given the higher levels of receptor expression at this time (57). Because Y79 cells represent primitive neuroectoderm (23), it is reasonable to assume that they would respond to insulin in a manner similar to a fetal neuron; i.e., differentiation and/or neurite outgrowth. To date however,the only observed response of Y79 cells to insulin is increased glycine uptake (55). The experiments reported here show that Y79 cells would be a useful cellular model of neural retinal cells for further investigation of the function and regulation of neuronal InsRs.

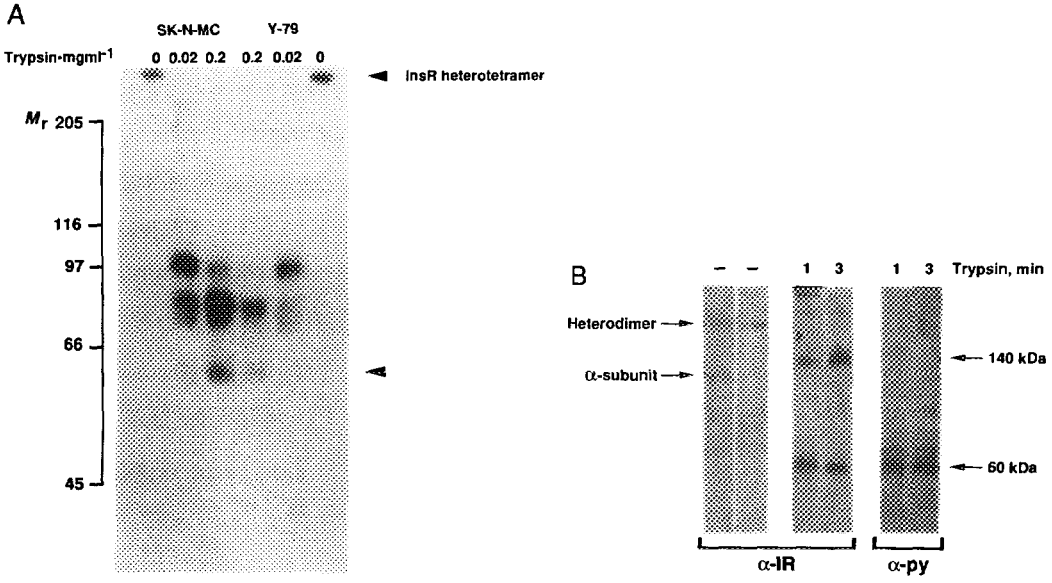

Figure 5. Trypsin activation of the InsR.

5A. Y79 cell membranes. 16 μg of WGA-agarose enriched membranes from Y79 or SK-N-MC cells was treated with TPCK-trypsin at the indicated concentrations for 1 min. The reaction was stopped and the activated receptors were incubated for 20 min with [γ-^{32}P]-ATP. Following immunoprecipitation with α-pY IgG, the samples were analyzed by non-reducing SDS-PAGE and autoradiography of the dried gel.

5B. Human placental membranes. 100 μg of WGA-agarose enriched human placental membrane protein was treated with 10 μg TPCK-trypsin for the indicated times. The reaction was stopped by addition of excess soybean trypsin inhibitor, followed by a 20 min incubation with 100 μM ATP. The samples were split in two, resolved by non-reducing SDS-PAGE, transferred to nitrocellulose and probed with α-InsR or α-pY IgG.

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