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Secretion of Annexin II via Activation of Insulin Receptor and Insulin-like Growth Factor Receptor*

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Annexin II is secreted into the extracellular environment, where, via interactions with specific proteases and extracellular matrix proteins, it participates in plasminogen activation, cell adhesion, and tumor metastasis and invasion. However, mechanisms regulating annexin II transport across the cellular membrane are unknown. In this study, we used coimmunoprecipitation to show that Annexin-II was bound to insulin and insulin-like growth factor-1 (IGF-1) receptors in PC12 cells and NIH-3T3 cells overexpressing insulin (NIH-3T3^{IR}) or IGF-1 receptor (NIH-3T3$^{IGF\text{-}1R}$). Stimulation of insulin and IGF-1 receptors by insulin caused a temporary dissociation of annexin II from these receptors, which was accompanied by an increased amount of extracellular annexin II detected in the media of PC12, NIH-3T3^{IR}, and NIH-3T3$^{IGF\text{-}1R}$ cells but not in that of untransfected NIH-3T3 cells. Activation of a different growth factor receptor, the platelet-derived growth factor receptor, did not produce such results. Tyrphostin AG1024, a tyrosine kinase inhibitor of insulin and IGF-1 receptor, was shown to inhibit annexin II secretion along with reduced receptor phosphorylation. Inhibitors of a few downstream signaling enzymes including phosphatidylinositol 3-kinase, pp60c-Src, and protein kinase C had no effect on insulin-induced annexin II secretion, suggesting a possible direct link between receptor activation and annexin II secretion. Immunocytochemistry revealed that insulin also induced transport of the membrane-bound form of annexin II to the outside layer of the cell membrane and appeared to promote cell aggregation. These results suggest that the insulin receptor and its signaling pathways may participate in molecular mechanisms mediating annexin II secretion.

Annexin II (AII)[1] belongs to a family of calcium-dependent phospholipid-binding proteins that are expressed in diverse tissues and cell types (1). It is found in cells as a 36-kDa monomer and a 94-kDa heterotetramer (AII_{94}) made of two 36-kDa monomers (AII_{36}) and two molecules of an 11-kDa (AII_{11}) S100 protein (2). Like all annexins, AII contains a conserved protein core domain that comprises four repeated segments of about 70 amino acids each and is resistant to limited proteolysis. The N terminus of AII contains, as identified by both *in vivo* and *in vitro* studies, a serine phosphorylation site (Ser-25) for protein kinase C, a tyrosine phosphorylation site (Tyr-23) for pp60-Src, and a binding site for AII_{11} (2–5). Phosphorylation of AII by protein kinase C at this domain has been shown to regulate interaction of the AII heavy chain with the AII light chain (6, 7) and to influence aggregation of chromaffin granules and lipid vesicles (8, 9), which may play a role in membrane trafficking events such as Ca^{2+}-dependent exocytosis (10, 11). On the other hand, tyrosine phosphorylation of AII *in vitro* by pp60c-Src has been shown to play a negative regulatory role in AII's binding to and bundling of F-actin and formation of the annexin II heterotetramer complex with plasma membrane (12). The C-terminal domain of AII contains binding sites for Ca^{2+}, phospholipids, and F-actin (8).

Initially identified as an intracellular molecule, AII has been implicated in the regulation of a variety of cellular processes including Ca^{2+}-evoked exocytosis. AII is highly enriched in chromaffin granule preparations, is able to aggregate chromaffin vesicles in a Ca^{2+}-dependent manner, and partially restores the secretory response in permeabilized chromaffin cells (14). AII-regulated exocytosis appears to be associated with formation of the heterotetramer AII_{94}. AII has also been suggested to play a role in endocytosis. It is associated with endosomal membranes and is one of the few proteins transferred from a donor to an acceptor endosomal membrane in an *in vitro* fusion assay (15). The binding of AII to endosomes appears to be Ca^{2+}-independent but requires an intact N-terminal domain (16). Other intracellular functions that involve AII include immunoglobulin transport (17) and ion channel activity (18).

In addition to its intracellular functions, AII is secreted into the extracellular environment in both soluble and membrane-bound forms (19). Although the detailed functions of extracellular AII are not fully understood, AII is known to interact with matrix proteins and specific proteases to regulate plasminogen activation, cell migration, and cell adhesion (20, 21). For example, AII acts as a receptor for the secreted serine proteases plasminogen and tissue plasminogen activator on the endothelial cell surface and thereby triggers generation of plasmin (20, 22). In addition, through interaction with extracellular matrix proteins such as tenascin-C (23) or certain collagens (24), AII appears to play a role in mediating cell focal adhesion, migration, and mineralization of growth plate cartilage. Further-

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[1] The abbreviations used are: AII, annexin II; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; IR, insulin receptor; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; PBS, phosphate-buffered saline; BiSM-1, bisindolylmaleimide I.

more, extracellular and membrane-bound AII may play a significant role in tumor invasion and metastasis. On the surface of metastatic lymphoma cells, AII enhances adhesion of these cells to liver sinusoidal endothelial cells (21). On the surface of tumor cells, the AII heterotetramer interacts with cathepsin B, a cysteine protease that is secreted into the extracellular environment and plays a prominent role in tumor development and invasion (25, 26). Interaction of AII with cathepsin B may facilitate a proteolytic cascade in the extracellular matrix that selectively degrades extracellular matrix proteins (27).

Because AII lacks a signal peptide and cannot be secreted through conventional endoplasmic reticulum secretory pathways, the mechanism(s) by which AII is secreted are currently unknown. Identification of molecules that participate in transporting AII to the extracellular matrix will be useful for understanding the role of AII in adhesion and mineralization. It was recently reported that Tyr phosphorylation sites located on the N terminus of AII can be phosphorylated by the insulin (IR) and the IGF-1 receptor (28, 29). In this study we report on the involvement of the IR signaling pathway in regulation of AII secretion.

EXPERIMENTAL PROCEDURES

Maintenance of Cell Culture—Normal NIH-3T3 cells (nontransfected mouse fibroblasts) and NIH-3T3 cells stably transfected with human insulin receptor (NIH-3T3^{IR}) or insulin-like growth factor-1 receptor (NIH-3T3$^{IGF\text{-}1R}$) (30, 31) were routinely cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (ICN Biomedical, Inc.) in 95% humidified air and 5% CO_2 at 37 °C. Rat PC12 cells (American Type Culture Collection, Manassas, VA) were maintained in poly-L-lysine (Sigma)-coated (10 μg/ml) T25 plastic flasks in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (Invitrogen), 5% fetal bovine serum (Hyclone Laboratories) and penicillin (100 units/ml) and streptomycin (100 μg/ml). For immunocytochemistry experiments, cells were cultured on poly-L-lysine-coated 10-mm glass coverslips (Carolina Biomedicals, Inc.).

Treatment of Cells with Insulin, Platelet-derived Growth Factor (PDGF), and Different Inhibitors—Once NIH-3T3, NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells were over 95% confluent in culture dishes, they were "starved" in serum-free Dulbecco's modified Eagle's medium overnight. The culture medium was replaced with fresh medium at about 15 min prior to insulin treatment. Cells were treated with different concentrations of insulin or PDGF (100 ng/ml) at 37 °C for 3 min for most experiments except for the time course experiment in which cells were treated with 100 nM insulin for various times ranging from 0 to 60 min. When treated with different inhibitors, cells were preincubated with 50 μM IR tyrosine kinase inhibitor tyrphostin AG1024 (Alexis Biochemicals) for 30 min, 100 nM PI 3-kinase inhibitor wortmannin (Sigma) for 90 min, PP1 (kindly provided by Dr. Anthony Bishop of Princeton University), and bisindolylmaleimide I (BiSM-1; Alexis Biochemicals) for 15 min. After preincubation, cells were given another dose of each inhibitor immediately prior to insulin treatment. At the end of the insulin or PDGF stimulation, the culture media were rapidly transferred to labeled tubes, to which a protease inhibitor mixture (Sigma) was added to a final concentration of 1%. Cells were briefly rinsed with precooled PBS and frozen by placing the dishes in liquid nitrogen. Cells were then thawed on ice and harvested in 200 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 1 mM vanadate and 1% protease inhibitor mixture (Sigma). After incubation on ice for 40 min, the cell lysate was centrifuged at 8,000 rpm for 3 min at 4 °C to pellet the remaining cellular debris. The supernatants were collected and kept at −70 °C for further biochemical experiments.

Immunoprecipitation and Immunoblotting—The interaction of AII with IR and IGF-1R was assessed by coimmunoprecipitation. In these experiments, IR or IGF-1R was immunoprecipitated from the cell lysate by incubating an equal amount of lysate (200 μg) with either anti-IR or anti-IGF-1R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody in lysis buffer at 4 °C on a rotating wheel overnight, followed by the addition of protein A-agarose and incubation for another 2 h. The immunocomplex was washed two times with lysis buffer and one time with washing buffer (20 mM Tris, pH 7.4, 150 mM NaCl). After boiling in 30 μl of SDS-PAGE sample buffer for 10 min, samples were resolved on a 4–20% SDS-PAGE gel (Novex, Inc.) and transferred onto a nitrocellulose membrane (Schleicher & Schuell). Following incubation with PBST (PBS plus 0.1% Tween 20) containing 5% low fat milk to block nonspecific binding sites, the membrane was incubated with an anti-AII antibody (Santa Cruz Biotechnology) at 4 °C overnight and then with a secondary antibody (Sigma). The immunoreactive signal was revealed in a chemiluminescent process using an ECL reagent (Pierce). To verify the protein-protein interaction, AII was also immunoprecipitated with anti-AII antibody, and the coprecipitated proteins were identified on a Western blot with anti-IR, anti-IGF-1R, or anti-phosphotyrosine antibody, respectively. In addition, the amount of protein immunoprecipitated by their own primary antibodies was routinely measured on Western blots.

Measurement of Protein Phosphorylations of IR, IGF-1R, Atk, Erk1/2, and AII36—To detect the activation of IR and IGF-1R in the cells treated with insulin, the cell lysate was immunoprecipitated with an anti-phosphotyrosine antibody (Py20; Santa Cruz Biotechnology Inc.), and the phosphorylated form of IR or IGF-1R from the immunoprecipitation was revealed by immunoblotting with either anti-IR or IGF-1R antibody (32). Alternatively, phosphorylation of these receptors was measured directly on Western blots with an anti-phospho-IR/IGF-1R antibody (Cell Signaling Technology) that specifically recognizes the Tyr(P)-1146 of IR and the Tyr(P)-1131 of IGF-1R. Similar methods were used for detecting phosphorylations of Akt and Erk1/2 with specific antibodies that recognize phospho-Akt (Ser-473) or phospho-Erk1/2. Levels of immunoreactive signals for phosphorylated proteins were then normalized against that for the total amount of each corresponding protein, which was revealed with an anti-regular form of the protein. Tyrosine phosphorylation of AII_{36} was examined by immunoprecipitation of AII with an anti-AII_{36} antibody, followed by detection of phospho-AII_{36} on Western blots with an anti-phosphotyrosine antibody (Py20).

Measurement of Extracellular AII_{36}—The overnight culture medium was replaced with fresh medium about 15 min prior to insulin treatment. Cells were stimulated with 100 nM of insulin at 37 °C for 3 min, and the culture medium was rapidly collected at the end of the reaction. Control cells were added in the same volume of lymphocyte buffer that was used to dilute insulin. The collected medium was centrifuged at 5000 rpm for 3 min to pellet any possible suspended cells or debris in the medium. The supernatant medium was collected, and the same volume of the medium from each condition was concentrated using the YM-10 Centricon (Millipore Corp.). After treatment with SDS-sample buffer, the same amount of concentrated sample was resolved on a 4–20% gradient SDS-PAGE gel, and the secreted AII_{36} was detected on Western blots by anti-AII_{36} antibody.

Immunocytochemistry—The nontreated and insulin-treated cells were fixed with 4% formaldehyde in PBS (pH 7.4) at room temperature for 5 min. After the cells were washed three times with PBS, they were permeabilized with 1% Triton-X100 in PBS (pH 7.4), and the nonspecific binding sites were blocked with 10% normal horse serum in PBS (pH 7.4). Cells were then double stained with either anti-AII/anti-IR or anti-AII/anti-IGF-1R antibodies at room temperature for 2 h. The dilution of these antibodies was 1:100–200. After the cells were washed three times with PBS, a secondary anti-goat IgG conjugated with Texas Red (Vector Laboratories, Inc.) and anti-rabbit IgG conjugated with fluorescein (Vector Laboratories) were added to the cells and incubated at room temperature for 1 h in the dark. The cells were then washed three times, sealed with VECTASHIELD (Vector Laboratories) and observed under a confocal microscope.

Data Analysis—The immunoreactive signals from Western blots were quantified using the NIH Image analyzing program. For experiments measuring protein phosphorylation using anti-phospho-protein antibodies, ratios of immunoreactive signals between the phosphorylated and total amounts of each particular protein were calculated. Signals from insulin-treated cells were converted to the percentage of the control cells, and values from at least three independent experiments were subjected to either a two-tailed t test or one-way analysis of variance. p values of less than 0.05 were considered statistically significant.

RESULTS

Coimmunoprecipitation of Annexin II with IR and IGF-1R—The NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells expressed similar levels of AII_{36} to those in normal NIH-3T3 cells (Fig. 1*A*), indicating that overexpression of IR or IGF1-R had no effect on the expression of AII_{36}. In the coimmunoprecipitation experiment, a substantial amount of AII_3 coimmunoprecipitated with both

IR and IGF-1R from NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells under nonstimulated conditions (*INS* −) (Fig. 1*B-1*). Once cells were treated with 100 nM insulin (*INS* +) for 3 min; however, the amount of AII_{36} "pulled down" by IR or IGF-1R was markedly reduced (Fig. 1*B-1*). Statistical analysis of data from three independent replications indicated a highly significant effect of insulin treatment ($p < 0.001$). The reduced amount of AII_{36} coimmunoprecipitated with IR and IGF-1R was not due to a decrease in the amount of IR and IGF-1R precipitated by the antibodies, since similar amounts of IR and IGF-1R were detected from the precipitated samples (Fig. 1*B-2*). This result was confirmed by a reversed immunoprecipitation experiment, in which an anti-AII antibody was used during precipitation, followed by detection of IR and IGF-1R on Western blots with anti-IR or anti-IGF-1R antibodies. Again, although a similar amount of AII_{36} was precipitated by the anti-AII antibody (Fig. 1*B-4*), significantly less IR and IGF-1R were coprecipitated with AII_{36} after insulin stimulation (Fig. 1*B-3*). In addition to the mature IGF-1R, the IGF-1R precursor with a relative molecular mass of 180-kDa was also "pulled down" with AII_{36}, suggesting that AII_{36} interacts with both the mature receptor and the precursor.

To test the specificity of IR/AII and IGF-1R/AII coprecipitation, we performed several control experiments in which the normal rabbit and goat IgG were employed in the immunoprecipitation. As shown in Fig. 1*C*, in neither NIH-3T3^{IR} nor NIH-3T3$^{IGF\text{-}1R}$ cells was AII_{36} "pulled down" by the normal rabbit IgG (Fig. 1*C-1*). Similarly, neither IR nor IGF-1R was found in precipitated samples using the normal goat IgG (Fig. 1*C-2*). We also examined another member of the annexin family, annexin VI, on Western blots following immunoprecipitation with anti-IR and anti-IGF-1R antibodies. As shown in Fig. 1*C-3*, annexin VI was expressed in both NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells with a higher abundance in the latter. The expression levels of annexin VI were not affected by INS treatment. Unlike AII_{36}, no annexin VI was coimmunoprecipitated with IR or IGF-1R (Fig. 1*C-4*). These results suggest that AII36 specifically interacts with IR and IGF-1R.

Coimmunoprecipitation of Annexin II with IR and IGF-1R from PC12 Cells—To test whether coprecipitation of AII_{36} with IR and IGF-1R from NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells was due to an effect of overexpression of these receptors, we next examined interactions of AII_{36} with endogenous IR and IGF-1R in rat PC12 cells. Unlike IR and IGF-1R that were overexpressed in NIH-3T3 cells, IR and IGF-1R were expressed in PC12 cells with markedly different abundance. Fig. 2*A* shows that levels of immunoreactive signals for IR from NIH-3T3^{IR} cells and of IGF-1R from NIH-3T3$^{IGF\text{-}1R}$ cells were closely correlated with amounts of the protein resolved on SDS-PAGE (*open circle*, NIH-3T3^{IR}; *filled circle*, NIH-3T3$^{IGF\text{-}1R}$). Since only a negligible level of immunoreactive signals for IR and IGF-1R was detected from nontransfected NIH-3T3 cells (Fig. 2*A*, *NIH-3T3-1* and *NIH-3T3-2*), signals detected by anti-IR and -IGF-1R antibodies from NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells should mainly reflect levels of each overexpressed receptor. In PC12 cells, the relative amount of IGF-1R was about 4 times higher than that of IR (*open square*, PC12-IR; *filled square*, PC12-IGF-1R). Consistent with that observed in NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells, a substantial amount of AII_{36} was coprecipitated with IR or IGF-1R from PC12 cells, which was also significantly decreased ($p < 0.001$) upon insulin treatment (Fig. 2*B*). It should be noted that although PC12 cells expressed high levels of IGF-1R, the relative amount of AII_{36} coprecipitated with IGF-1R under basal conditions was significantly lower ($p < 0.001$) than that with IR.

Insulin-induced Increase in Extracellular AII_{36}—AII_{36} is known to be secreted into the extracellular matrix as both soluble and membrane-bound forms, although the mechanism(s) and molecular pathway(s) underlying its secretion have not been identified. In order to understand the physiological significance of AII/IR and AII/IGF-1R interactions and subsequent molecular events of the disassociation of AII from these receptors, we investigated AII secretion from the NIH-3T3, NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells. As shown in Fig. 3*A*, no AII_{36} was detected in the culture medium from untransfected NIH-3T3 cells under both basal conditions and after insulin stimulation. In the NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells, however, AII_{36} was found in the culture medium. The amount of AII_{36} in the culture media from these cells was markedly increased following insulin stimulation ($p < 0.001$, *t* test) under those conditions. Fig. 3, *B-1* and *B-3*, shows activation of IR and IGF-1R by insulin in a dose-dependent manner as indicated by the extent of tyrosine phosphorylation of the receptors. These receptors were not activated by PDGF. When treated with insulin for 3 min, the amount of extracellular AII_{36} from NIH-3T3^{IR} cells was also shown to be insulin dose-dependent and correlated with phosphorylation levels of IR. The insulin-induced extracellular AII_{36} from NIH-3T3$^{IGF\text{-}1R}$ cells, however, remained at a similar level when insulin concentration was increased from 1 to 100 nM (Fig. 3*B-4*). No AII_{36} was detected in the culture media from the PDGF-treated NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells (Fig. 3, *B-2* and *B-4*). In addition, no annexin VI was detected in the culture media under similar conditions (data not shown). When cells were treated with the IR tyrosine kinase inhibitor AG1024, phosphorylation of IR and IGF-1R were both inhibited (Fig. 3*C-1*), along with a substantial reduction of the amount of AII_{36} in the culture media (Fig. 3*C-2*). However, when cells were treated with the PI 3-kinase inhibitor wortmannin, which abolished the insulin-induced activation of Akt (Fig. 3*D*), neither the insulin-induced phosphorylation of IR and IGF-1R nor the insulin-induced increase in extracellular AII_{36} was affected (Fig. 3*C*). These results suggest that insulin-induced secretion of AII_{36} from IR- and IGF1R-overexpressed cells as well as PC12 cells.

Time Course of Insulin Effects on Receptor Phosphorylation, Protein Interaction, and AII Secretion—A time course study of insulin was performed to further investigate the insulin-induced AII secretion and the receptor/AII association. NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells were treated with 100 nM insulin for various lengths of time, and phosphorylation of IR and IGF-1R was examined on Western blots by an anti-phospho-IR/IGF-1R antibody. As shown in Fig. 4*A*, insulin triggered a rapid phosphorylation of IR and IGF-1R at 3 min after treatment. The phosphorylation of IR remained at similarly high levels for as long as 60 min after treatment, whereas phosphorylation of IGF-1R showed a time-dependent increase that peaked at 30 min post-treatment and remained high at 60 min post-treatment. Whereas the amount of AII_{36} coprecipitated with IR and IGF-1R was markedly decreased at 3 min after insulin treatment, it returned to the control level 15 min after insulin treatment (Fig. 4*B*). Secretion of AII36 from NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells was detected at all times examined after insulin stimulation, although it was slightly reduced from 30 min onward after insulin treatment (Fig. 4*C*).

Effects of c-Src and Protein Kinase C Inhibitors on Insulin-stimulated AII Secretion and Interaction—Since AII is a major substrate of both pp60c-Src and protein kinase C, to investigate possible downstream involvement of these protein kinases in insulin-stimulated AII_{36} secretion, we applied, respectively PP1, a c-Src inhibitor, and BiSM-1, a cell-permeable protein kinase C inhibitor, to NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells 15

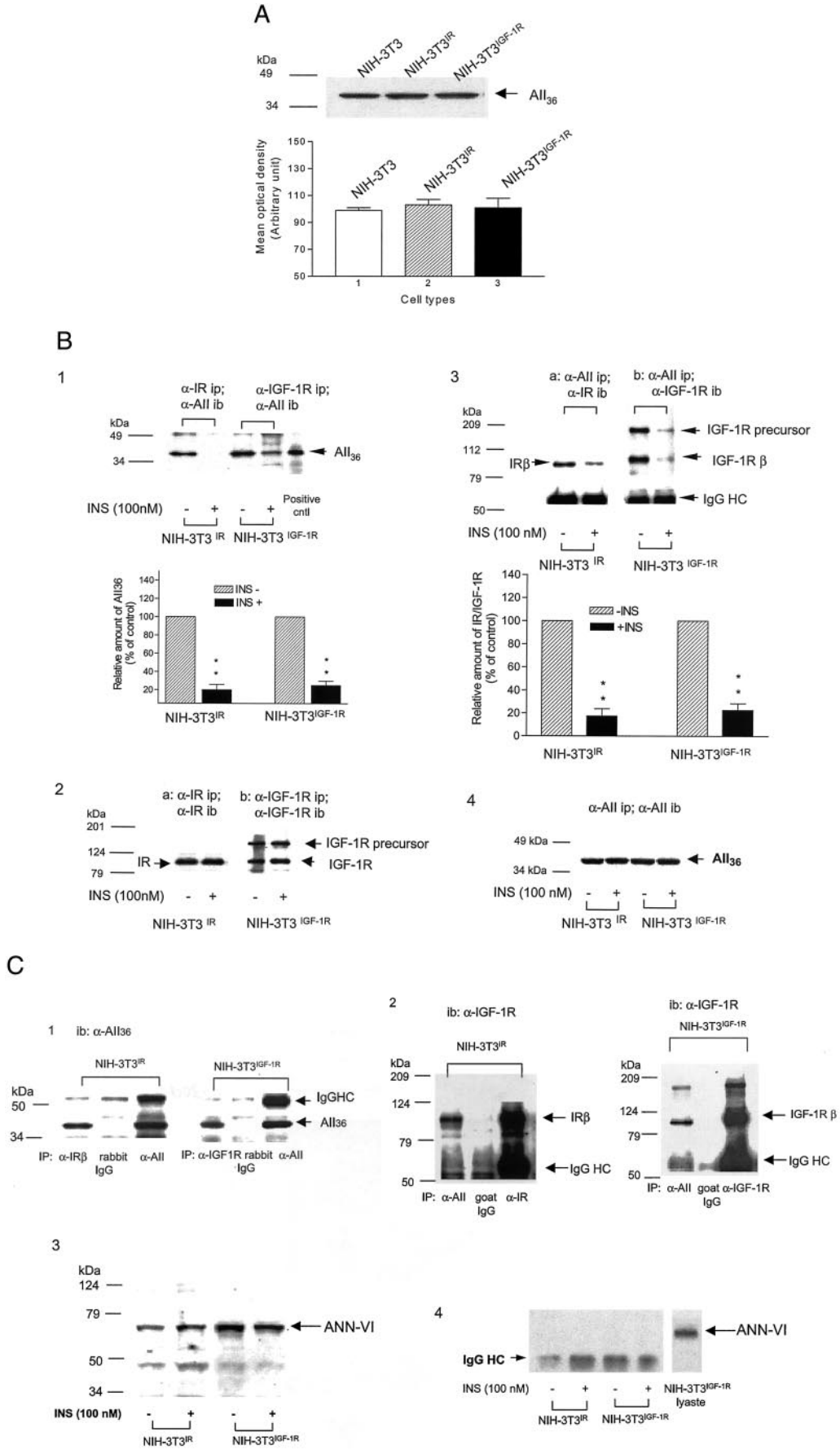

FIG. 1. *A*, expression of AII36 in NIH-3T3 cells. Similar amounts of cell lysates from NIH-3T3, NIH-3T3^{IR}, and NIH-3T3$^{IGF\text{-}1R}$ cells were resolved on 4–25% SDS gradient gels followed by detection of AII_{36} signals with an anti-AII antibody on a Western blot. No difference in expression of AII_{36} was observed across these cell types. *B*, interactions of AII36 with IR and IGF-1R. IR and IGF-1R from nonstimulated and INS-treated NIH-3T3^{IR}

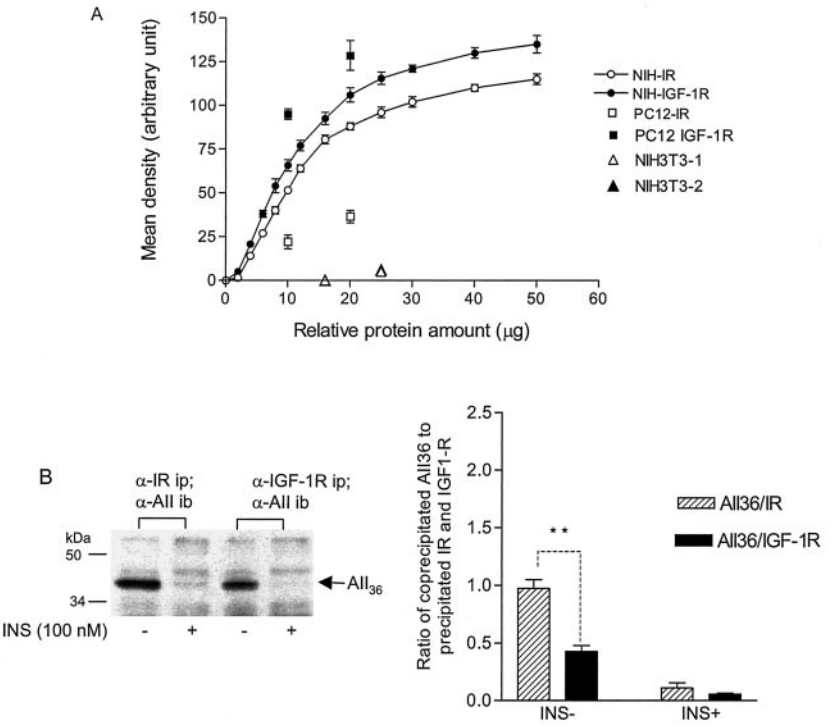

FIG. 2. *A*, relative abundance of IR and IGF-1R in PC12 cells. The amount of IR and IGF-1R in PC12 cells were assessed, respectively, by measuring the immunoreactive signal levels on Western blots against a concentration curve of the corresponding receptor overexpressed in NIH-3T3 cells. *Open circles*, IR immunoreactive signals in NIH-3T3^{IR} cells; *filled circles*, IGF-1R immunoreactive signals in NIH-3T3$^{IGF\text{-}1R}$ cells. *Open squares*, IR signals in PC12 cells; *filled squares*: IGF-1R signals in PC12 cells; *open triangles*, IR signals in nonreceptor-expressed NIH-3T3 cells; *filled triangle*, IGF-1R signals in nonreceptor-expressed NIH-3T3 cells. *B*, coimmunoprecipitation of AII$_{36}$ with IR and IGF-1R from PC12 cells. PC12 cell lysates with and without INS stimulation were subjected to immunoprecipitation with either the anti-IR or anti-IGF-1R (2 μl/ml) antibody, followed by detection of AII$_{36}$ on Western blots. Densitometric values for coprecipitated AII$_{36}$ with IR or IGF-1R were normalized, respectively, against that for each precipitated receptor. The *bar* and *line graphs* (mean ± S.E.) summarize results from at least three repeated experiments. **, $p < 0.001$, *t* test.

min prior to insulin treatment. PP1 inhibited insulin-induced phosphorylation of the extracellular signal-regulated kinase (Erk1/2), indicating the effectiveness of the inhibitor (Fig. 5*A*). BiSM-1 was also effective in abolishing phosphorylation of Erk1/2 in human fibroblasts (data not shown), a downstream event of bradykinin receptor activation (33). These two inhibitors, however, had no effects on insulin-stimulated phosphorylation of IR (Fig. 5*B*) and IGF-1R (Fig. 5*C*); nor did they affect the subsequent AII secretion (Fig. 5, *D* and *E*) and dissociation of AII from IR and IGF-1R (data not shown). Tyr phosphorylation of AII$_{36}$ was assessed by immunoprecipitation of AII$_{36}$ with an antibody that interacts with the C terminus of AII$_{36}$. The *in vivo* Tyr phosphorylation of AII$_{36}$ was then examined on Western blots with an anti-phospho-Tyr antibody. An apparent basal level of AII$_{36}$ Tyr phosphorylation was detected in all cells examined before insulin treatment, but this Tyr phosphorylation was significantly decreased ($p < 0.001$) after insulin stimulation (Fig. 5*F*). PP1 showed effects on neither the basal AII$_{36}$ phosphorylation nor the changes in AII$_{36}$ phosphorylation under insulin treatment (Fig. 5*G*). These results suggest that the insulin-induced AII secretion may be a direct consequence of receptor activation that does not involve downstream kinases such as protein kinase C and c-Src.

Immunocytochemistry Results—Fig. 6 shows the immunocytochemistry results, in which cells were double stained with either IR or IGF-1R (*green*) and AII$_{36}$ (*red*) antibodies. *Images* in Fig. 6*A* show double staining of IGF-1R and AII$_{36}$ in NIH-3T3$^{IGF\text{-}1R}$ cells, whereas *images* in Fig. 6*B* show double staining of IR and AII$_{36}$ in PC12 cells. Under nonstimulated conditions, AII$_{36}$ was homogeneously distributed in the cytosolic compartment. Prominent colocalization of AII$_{36}$ with both IR and IGF-1R was seen in both cells indicated by the *yellow color* (Fig. 6, *A-1* and *B-1*). Upon insulin stimulation, a reduction of intracellular AII$_{36}$ levels was seen in both NIH-3T3$^{IGF\text{-}1R}$ and PC12 cells alone with decreased colocalization of AII$_{36}$ with IGF-1R and IR (Fig. 6, *A-2* and *B-2*). In addition, an insulin-induced increase in the amount of AII$_{36}$ was detected in the extracellular space. The extracellular AII$_{36}$ appeared to attach to the outside surface of the membrane, particularly in the NIH-3T3$^{IGF\text{-}1R}$ cells (Fig. 6*B-2*, *pointed arrow*). Fig. 6*B-3* shows the enlarged area of the *square* in Fig. 6*B-2*. Similar results were also observed in NIH-3T3^{IR} cells (data not shown). Western blotting showed consistent findings that insulin stimulation induced a reduction in intracellular AII$_{36}$ in a dose-dependent manner (Fig. 6*C*).

DISCUSSION

Although it has long been known that AII$_{36}$ can be secreted into the extracellular compartment, the molecular mechanisms underlying secretion of AII$_{36}$ are unknown. Our results show that insulin stimulation markedly increased the amount of extracellular AII$_{36}$, suggesting a link between IR/IGF-1R signaling and AII$_{36}$ secretion. AII$_{36}$ is known as one of the major substrates of c-Src protein-tyrosine kinase (3, 12) and is also involved in signaling events downstream from IR (27). Under nonstimulated conditions, AII$_{36}$ was associated with IR and IGF-1R as indicated by coimmunoprecipitation of AII$_{36}$ with IR and IGF-1R from NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells.

and NIH-3T3$^{IGF\text{-}1R}$ cells were immunoprecipitated (*ip*) with anti-IR and anti-IGF-1R antibody, respectively. The coprecipitated AII$_{36}$ was detected with anti-AII antibody (*B-1*). The amount of precipitated IR and IGF-1R was detected on Western blots (*ib*) with anti-IR or anti-IGF-1R antibody (*B-2*). Immunoprecipitation was also performed with anti-AII antibody followed by detection of the amount of coprecipitated IR and IGF-1R proteins (*B-3*) and the amount of precipitated AII$_{36}$ (*B-4*) on Western blots. The *bar graphs* in all *panels* summarize results from at least three independent replicates (mean ± S.E.; **, $p < 0.001$, *t* test). *C*, negative control experiments. *1*, cell lysates were precipitated, respectively, with anti-IR (or anti-IGF-1R), normal rabbit IgG, and anti-AII36. The precipitated samples were then measured for AII$_{36}$ on Western blots. *2*, cell lysate was precipitated with anti-AII, normal goat IgG, and anti-IR (or anti-IGF-1R) antibodies, followed by detection of IR/IGF-1R on Western blots. *3*, expression of annexin VI in NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells. Similar amounts of cell lysates with and without INS stimulation were resolved in SDS-PAGE. Immunoreactive signal for annexin VI was measured on Western blots using a specific anti-annexin VI antibody. *4*, the same cell lysates were subjected to immunoprecipitations with anti-IR or IGF-1R antibody. The precipitated samples were resolved on SDS-PAGE and blotted with anti-annexin VI antibody. No annexin VI was detected from the immunoprecipitated samples. *IgG HC*, IgG heavy chain.

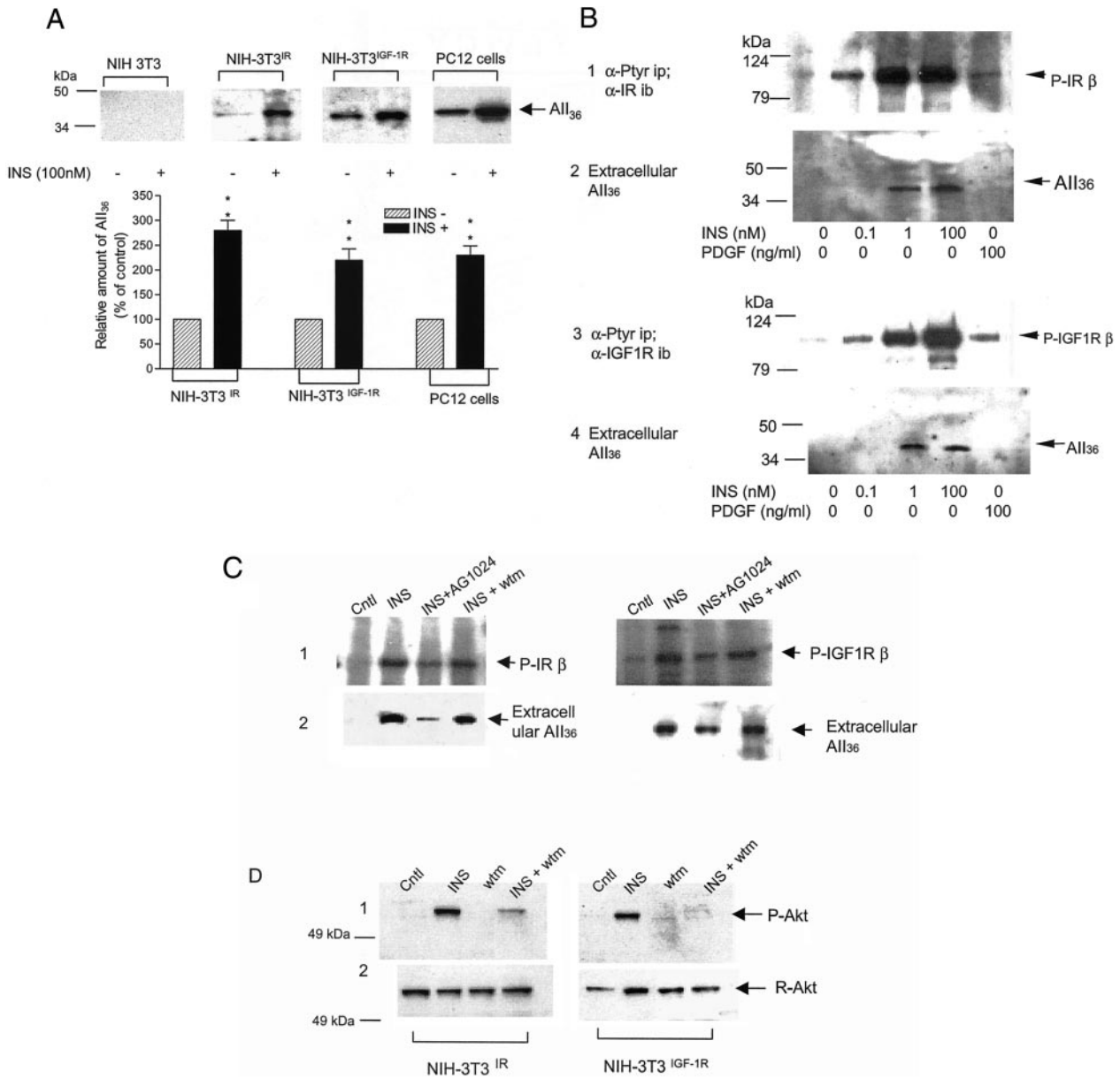

FIG. 3. *A*, insulin-induced changes in the amount of extracellular AII_{36}. The NIH-3T3, NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells were treated with (+) or without (−) insulin at 37 °C for 3 min. AII_{36} from the concentrated culture medium was dissolved on SDS-PAGE and detected on Western blots. No AII_{36} secretion was observed in normal NIH-3T3 cells. Substantial increases in AII secretion were detected in NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells after insulin stimulation. The *bar graphs* indicate data (mean ± S.E.) from three replicates ($p < 0.001$, *t* test). *B*, dose-dependent effect of insulin on Tyr phosphorylation of IR/IGF-1R and secretion of AII36. In *1* and *3*, NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells were treated with different concentrations of insulin or 100 ng of PDGF at 37 °C for 3 min. The Tyr phosphorylation extent of IR and IGF-1R was measured by immunoprecipitating the phosphorylated receptors with an anti-phosphotyrosine antibody. The precipitated phospho-IR and -IGF-1R were then detected on Western blots with anti-IR or IGF-1R antibody. In *2* and *4*, the culture medium was collected after treatment with different insulin concentrations, and the secreted AII_{36} was detected on Western blots with an anti-AII36 antibody. *C*, effect of tyrphostin AG1024 and wortmannin on Tyr phosphorylation of IR, IGF-1R, and AII36 secretion. The NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells were pretreated with AG1024 or wortmannin prior to insulin stimulation. The Tyr phosphorylations of IR and IGF-1R (*3C-1*) as well as the extracellular AII36 (*3C-2*) were measured as described for *B*. *INS*, insulin; *wtm*, wortmannin; *AG1024*, tyrphostin AG1024; *P-IR*, phosphorylated IR; *P-IGF-1R*, phosphorylated IGF-1R. *D*, effect of wortmannin on the insulin-induced phosphorylation of Akt. The NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells were treated with or without 100 nM insulin in the presence or absence of 100 nM wortmannin. The insulin-stimulated phosphorylation of Akt was detected on Western blots with an anti-phospho-Akt antibody (*3D-1*). The total amount of Akt was measured with an anti-regular Akt antibody (*3D-2*).

Stimulation of cells with insulin markedly reduced association of AII_{36} with these protein-tyrosine kinases along with decreased tyrosine phosphorylation of AII_{36}. In the PC-12 cells, although IGF-1R was shown to be 4 times as abundant as the IR, a similar amount of AII was coprecipitated by both IR and IGF-1R antibody (Fig. 1, *D-1*). This may be due to the possibility that only a proportion of the receptors were associated with AII_{36} among the precipitated IGF-1R receptors from PC12 cells. Such factors as differences in binding affinity to AII between IR and IGF-1R in PC12 cells may also account for the observed results.

The insulin-stimulated dissociation of AII_{36} from IR or IGF-1R was shown to be a rapid but temporary event, since the amount of AII_{36} "pulled down" with the receptors returned to the control level 15 min after insulin stimulation, although phosphorylation of IR and IGF-1R lasted as long as 60 min after insulin stimulation. It should be noted that, in addition to the mature IGF-1R β-subunit, AII_{36} was also associated with

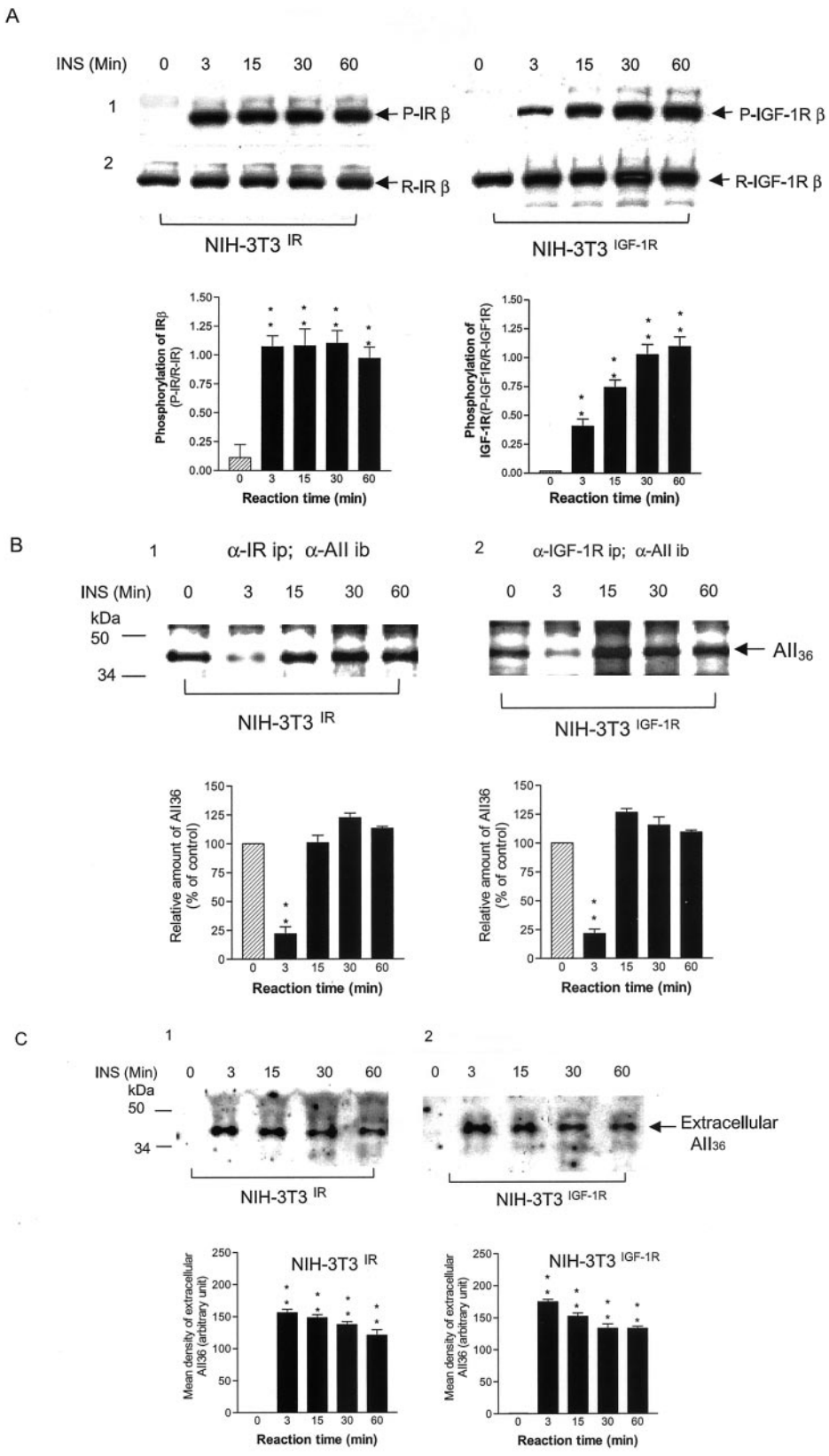

FIG. 4. *A*, time course effects of the insulin-induced receptor phosphorylation. The NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells were incubated with 100 nM insulin, and the reactions were terminated at different times ranging from 0 (negative control) to 60 min post-treatment. The insulin stimulated a prolonged receptor phosphorylation measured by an anti-phospho-IR/IGF-1R antibody on Western blots (*4A-1*). The extent of phosphorylation of IR and IGF-1R was assessed by calculating the ratio of the phospho-IR/IGF-1R to the total amount of each receptor measured by a regular anti-IR or -IGF-1R antibody (*4A-2*). *B*, time course effects of receptor/AII interaction after insulin stimulation. Cells were treated with insulin as described for *A*, and the receptor/AII_{36} interaction was examined as described in the legend to Fig. 1*B*. *C*, time course effects of the insulin-induced secretion of AII. After treatment with insulin as described for *A*, the extracellular AII36 from both NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells was measured as described in the legend to Fig. 3*A*. In all *panels* the bar graphs summarize data from four replications (mean ± S.E.; **, $p < 0.001$; one-way analysis of variance).

the IGF-1R precursor protein (Fig. 1*B-3*), which is a single polypeptide that contains α- and β-subunits of the receptor with a relative molecular mass of 180 kDa. IGF-1R precursor has been known to exhibit both ligand-binding ability and tyrosine kinase activity (34, 35). The insulin-stimulated IGF-1R precursor phosphorylation was also observed in the present study (Fig. 3*C-2*). Like the mature receptors, however, the physiological significance of the AII/IGF-1R precursor interaction remains to be determined.

Interestingly, the dissociations of AII_{36} from IR and IGF-1R were correlated with the insulin-stimulated increases in extracellular AII_{36} and a reduction in intracellular AII_{36}. The extracellular AII_{36} detected in the culture medium was not due to the contamination of intracellular AII_{36}, since all culture media

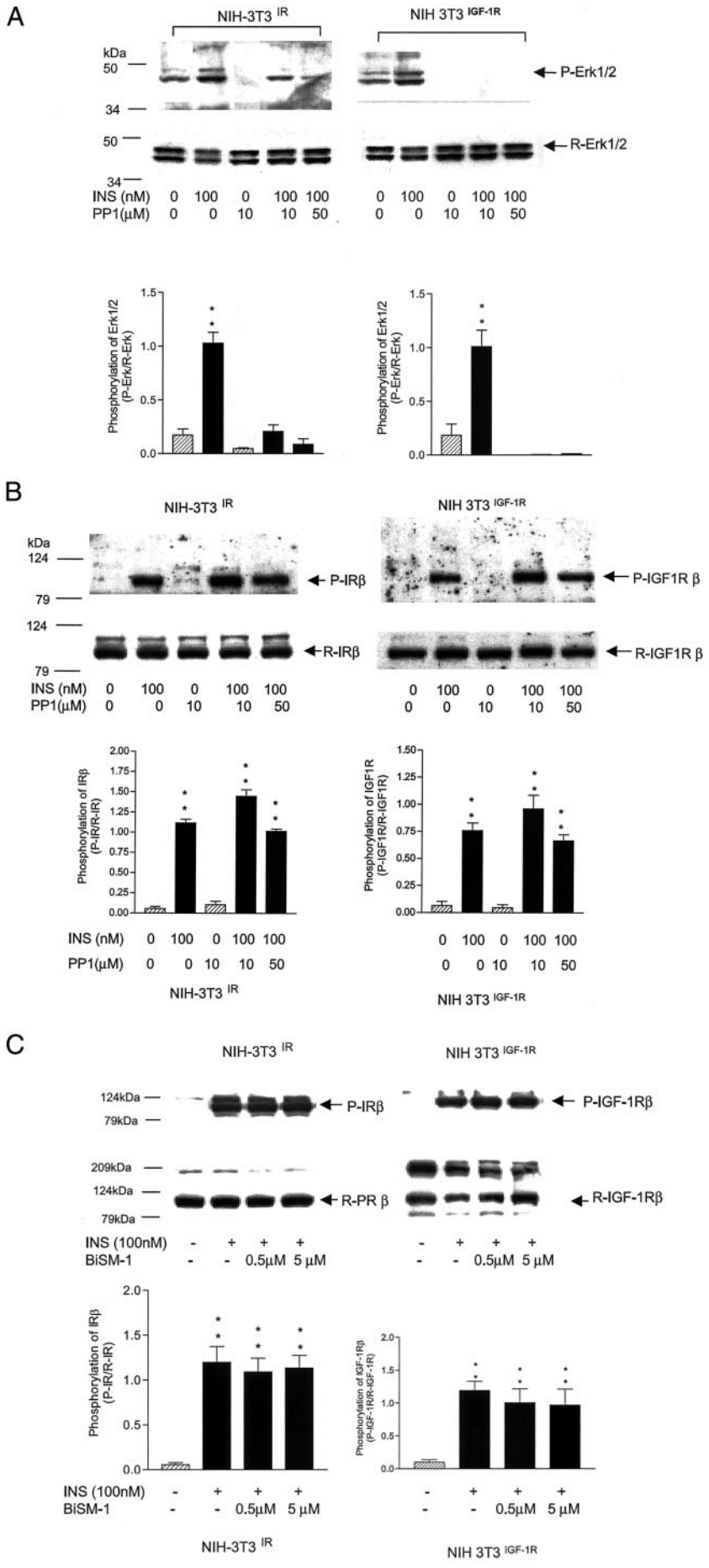

FIG. 5. *A*, effects of PP1 on insulin-stimulated Erk1/2 phosphorylation. Cells were treated with or without 100 nM insulin for 3 min at 37 °C in the presence or absence of different concentrations of PP1. The basal and the insulin-stimulated Erk1/2 phosphorylation were measured by an anti-phospho-Erk1/2 antibody (*P-Erk1/2*). The extent of phosphorylation was assessed by calculation of the ratio of phospho-Erk1/2 over the total amount of Erk1/2 (*R-Erk1/2*). *B*, effects of PP1 on insulin-stimulated IR/IGF-1R phosphorylation. Cells were treated under the same conditions as described for *A*. The insulin-activated phosphorylations of IR and IGF-1R were measured by anti-phospho-IR/IGF-1R antibody (*P-IR* and *P-IGF-1R*). The total amount of IR and IGF-1R was determined by using the regular anti-IR or anti-IGF-1R antibodies (*R-IR* and *R-IGF-1R*). Ratios of phosphorylated receptors over the total amount of the receptors were calculated to determine the extent of receptor phosphorylation. *C*, effects of BiSM-1 on insulin-stimulated IR/IGF-1R phosphorylation. Cells were treated with or without insulin in the presence or absence of different concentrations of BiSM-1 (0.5–5.0 μM). The resulting receptor phosphorylation was examined and analyzed as described for *B*. *D*, effects of PP1 on insulin-stimulated AII_{36} secretion. Cells were treated as described for *B*, and the extracellular AII_{36} was measured as described in the legend to Fig. 3*A*. *E*, effects of BiSM-1 on insulin-stimulated AII_{36} secretion. Cells were treated as described for *C*, and the extracellular AII_{36} was measured as described in the legend to Fig. 3*A*. *F*, changes in AII_{36} tyrosine phosphorylation after insulin stimulation. AII_{36} from nonstimulated and insulin-stimulated NIH-3T3^{IR}, NIH-3T3$^{IGF\text{-}1R}$, and PC12 cells were precipitated by anti-AII antibody, and its Tyr phosphorylation was detected in immunoblot (*ib*) with an anti-phospho-Tyr antibody. *G*, changes in AII Tyr phosphorylation after insulin treatment in the presence of PP1 were measured as described for *F*. The values of Tyr phosphorylation signals from each condition were normalized with the total amount of AII_{36} detected on Western blot with anti-AII antibody. In all *panels*, *bar graphs* summarize results from three or four experimental replicates (means ± S.E.; **, $p < 0.001$; *t* test and one-way analysis of variance).

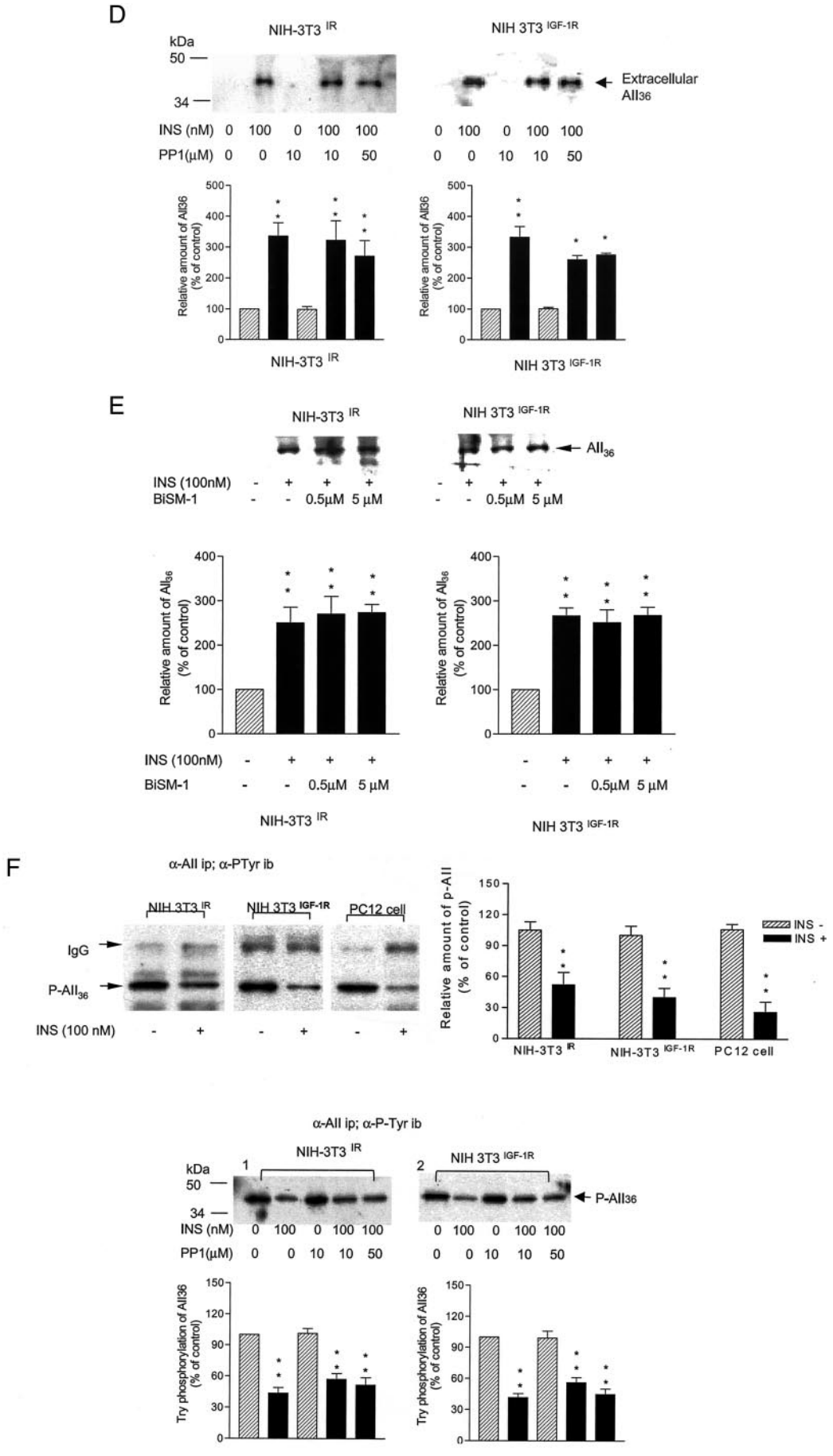

FIG. 5—*continued*

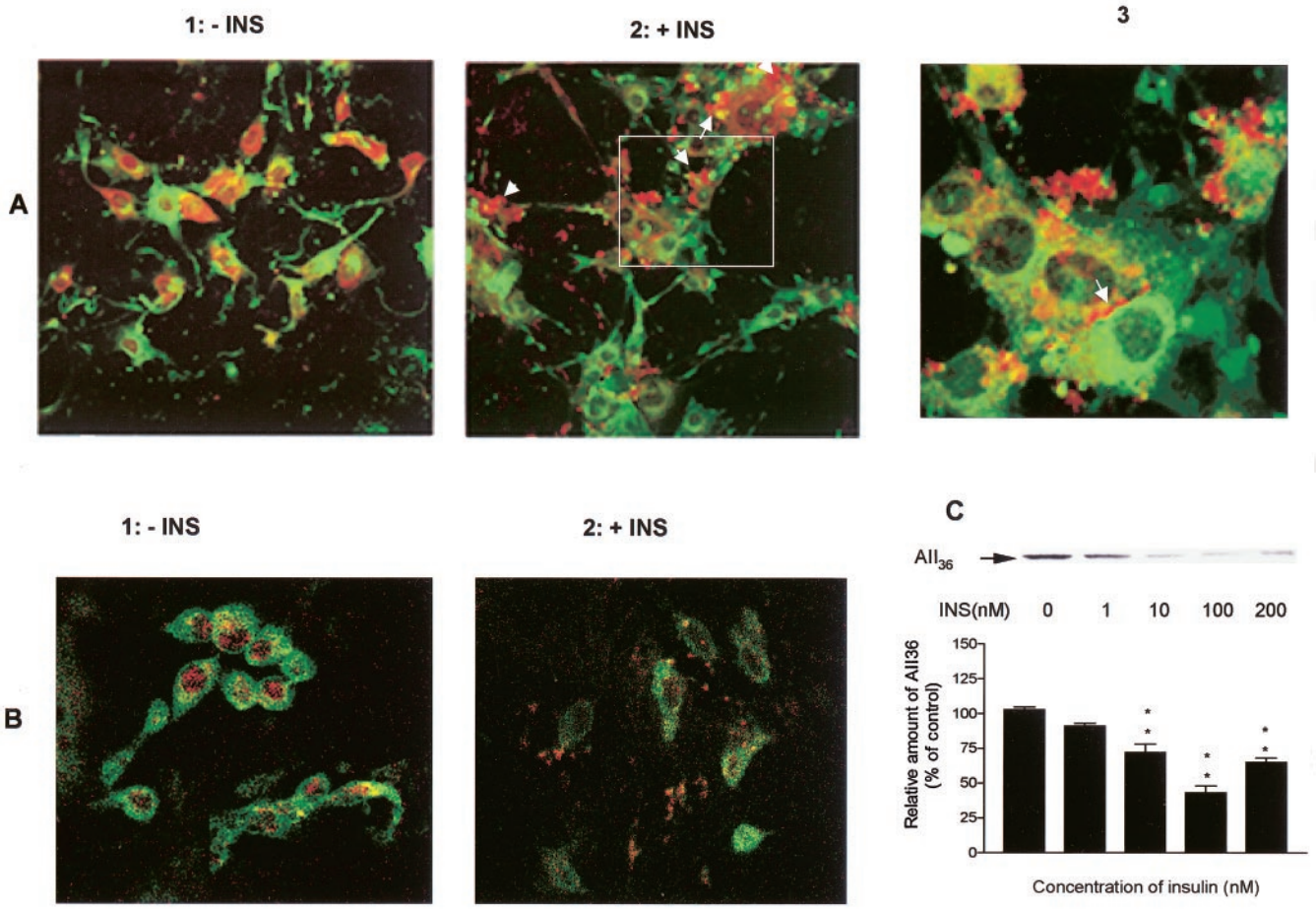

FIG. 6. **Immunocytochemistry staining performed in the NIH-3T3$^{IGF\text{-}1R}$ and PC12 cells.** *A*, NIH-3T3$^{IGF\text{-}1R}$ cells were stained with anti-IGF-IR and anti-AII antibodies before (*1*) and after (*2*) insulin stimulation. The immunoreactive signals were revealed by the addition of the secondary antibodies labeled with different fluorescent dyes. The green fluorescence represents IGF-1R signals and the red fluorescence represents AII_{36} signals. The *yellow* indicates colocalization of IGF-1R and AII36. The *arrows* in *2* point to AII bound to the outside surface of the cell membrane after insulin stimulation. *3*, an enlargement of field indicated by the *square* in *2*. *B*, PC12 cells were stained with anti-IR and anti-AII_{36} antibodies before (*1*) and after (*2*) insulin stimulation. The IR and AII36 signals were revealed with fluorescent secondary antibodies. *Green*, IR signals; *red*, AII_{36}. *C*, changes in intracellular AII36 after insulin stimulation. PC12 cells were treated with different concentrations of insulin. The cell lysates were resolved on SDS-PAGE, and the amount of intracellular AII_{36} was measured on Western blots with an anti AII_{36} antibody. The *bar graph* represents results from three replicates. **, $p < 0.001$.

had been carefully centrifuged to remove any suspending cells before concentration. Furthermore, extracellular AII_{36} was not seen in the nontransfected NIH-3T3 cells, which expressed the same amount of AII as NIH-3T3^{IR} and NIH-3T3$^{IGF\text{-}1R}$ cells.

The increases in the extracellular AII_{36} were insulin dose-dependent (particularly in NIH-3T3^{IR} cells) and were closely correlated with the tyrosine phosphorylation levels of IR and IGF-1R, an indication of the receptors' activation. The fact that a similar amount of AII was detected in the extracellular compartment of NIH-3T3$^{IGF\text{-}1R}$ cells after 1 and 100 nM insulin treatment might suggest that secretion of AII_{36} saturates at the activation level of IGF-1R in response to 1 nM of insulin. Alternatively, as shown by the immunocytochemistry results, a part of AII_{36} secreted from NIH-3T3$^{IGF\text{-}1R}$ cells under 100 nM insulin stimulation binds to the outer layer of the plasma membrane, which may have offset the amount of unbound AII_{36} detected in the culture medium. Inhibition of IR and IGF-1R kinase activities by AG1024 significantly reduced the insulin-stimulated extracellular AII_{36}. The fact that a more obvious reduction of extracellular AII_{36} was seen with IR inhibition compared with the IGF-1R inhibition suggests that transport of AII to the extracellular compartment may be more closely associated with activity of IR. Furthermore, activation of other growth factor receptors such as the PDGF receptor resulted in no extracellular AII_{36}. All of these results indicate that production of the extracellular AII_{36} is closely correlated with activities of IR and IGF-1R.

Since extracellular AII_{36} was detectable at all of the post-insulin treatment times and since the insulin-stimulated receptor phosphorylation was also persistent, it is not clear whether the detected AII_{36} after the longer term insulin stimulation was due to continuous secretion or was from a one-time secretion at an early stage of the stimulation. Given the fact that association of AII with IR and IGF-1R returned to control levels 15 min after insulin treatment, it is possible that AII_{36} is secreted shortly after activation of IR or IGF-1R and stays in the extracellular matrix until it is degraded by extracellular proteolysis. The reduction of extracellular AII_{36} at 30 and 60 min after insulin treatment may reflect such degradation.

In rat-1 fibroblasts overexpressing the human insulin receptor, insulin has been reported to induce rapid cytoskeletal protein rearrangement and membrane ruffling that requires activity of PI 3-kinase (36). One could argue that the increased extracellular AII_{36} after insulin stimulation might be a non-specific result of an insulin-induced loss of actin stress fibers and membrane ruffling in the IR- or IGF-1R-overexpressed cells. To test this possibility, we treated cells with the PI 3-kinase inhibitor wortmannin, which has been shown to prevent insulin-induced stress fiber breakdown (36). Treatment of cells with wortmannin did not reduce the insulin-stimulated

increase in extracellular AII_{36} but completely abolished the insulin-induced activation of Akt (Fig. 2*D*), another insulin-dependent molecular event downstream of PI 3-kinase (13, 37). These results suggest, therefore, that the insulin-induced extracellular AII_{36} is not due to cell membrane damage but rather a receptor activity-driven secretion process.

Although AII_{36} is known to be a major substrate for protein kinase C and pp60c-Src tyrosine kinase, inhibition of these two kinases did not affect the insulin-stimulated AII secretion and changes in its receptor interaction. It is possible, therefore, that in response to insulin stimulation, IR and IGF-1R directly regulate AII_{36} tyrosine phosphorylation through changes in their interaction with AII_{36}. Thus, secretion of AII_{36} might be an immediate cellular event coupled to IR and IGF-1R activation.

The increased extracellular AII_{36} after insulin stimulation was not only detected in the culture medium, which should contain the soluble form of AII_{36}, but also was seen on the outside layer of the plasma membrane, particularly in the NIH-3T3$^{IGF\text{-}1R}$ cells (Fig. 6*A*). Given that 1) IR and IGF-1R were shown to be associated with AII_{36} but not with other annexins such as annexin VI; 2) no extracellular annexin VI was detected under both nonstimulated and insulin-stimulated conditions; and 3) the increase in extracellular AII_{36} closely coincided with the reduction of the intracellular AII and the IR/AII_{36} and IGF-1R/AII_{36} shortly after insulin stimulation, it is tempting to speculate that IR and/or IGF-1R binds to AII_{36} and thereby anchors AII to the vicinity of the plasma membrane. Activation of IR or IGF1-R may regulate the secretion process of AII_{36} that requires dissociation of the bound AII_{36} from the receptors and Tyr dephosphorylation of AII. However, further research will be required to understand this process and the molecular mechanisms that underlie secretion of AII and to verify the role of IR and/or IGF-1R in secretion of AII.

Extracellular AII has been associated with cell adhesion and migration in both normal and malignant tumor cells through its interactions with extra matrix proteases and structural proteins. The present results may shed light on the potential role(s) of the insulin and the insulin-like growth factor signaling pathways in protein secretion, cell adhesion, and migration of both normal and malignant cells. Furthermore, given its roles in these extracellular events, AII may provide a potential therapeutic target for extracellular matrix-associated pathological processes such as tumor metastasis.

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Secretion of Annexin II via Activation of Insulin Receptor and Insulin-like Growth Factor Receptor

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