Annexins and Heparan Sulfate Proteoglycans in Pancreatic Endocrine β Cells

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Abstract

We previously demonstrated that annexins are localized in exocrine tissues such as kidney, liver, intestine and pancreatic acinar cells. We also elucidated the glycosaminoglycan (GAG) binding properties of annexins IV, V and VI and showed that each of them has its own specific binding activities to GAGs. In this study, we examined the function of annexin VI and GAGs in pancreatic β cells. Immunohistochemical studies and immunoblot assays showed that annexin VI localized in rat pancreatic islets, HIT-T15 cells derived from rat insulinoma and MIN6 cells derived from mouse insulinoma. Immunoblot assay detected heparan sulfate proteoglycan in those cell lysates but not chondroitin sulfate proteoglycan. Affinity adsorption assay showed annexin VI bound to the heparan sulfate proteoglycans derived from HIT-T15 cells indicating that these proteoglycans the endogenous ligand of annexin VI in β cells. Insulin secretion assay from permeabilized MIN6 cells showed that full-length human annexin VI promotes insulin release, but not other mutants. Inhibition of Ca^{2+}-dependent insulin release by GAGs was inferred that GAGs blocked binding of annexin VI and membrane vesicle. These results suggest that annexin VI was considered to exit in pancreatic endocrine cells, and to interact with both cell-extracellular matrix heparan sulfate proteoglycan and membrane vesicle in β cells.

1. Introduction

Annexins are a family of structurally related proteins that bind to phospholipids in a calcium-dependent manner. They consist of four (or eight in the case of annexin VI) conserved repeating structures of 70 amino acid residues and have an N-terminal domain which is highly variable in both sequence and length, that distinguishes different family members. Their exact biological functions have not yet been completely elucidated,

although a number of *in vitro* experiments have indicated that some annexins exhibit membrane channel activity, inhibit phospholipase A_2 and blood coagulation, function in regulation of membrane traffic and exocytosis and transmit intracellular signals as a kinase substrate [1-4].

We previously demonstrated that annexins are localized in exocrine tissues such as kidney, liver, intestine and pancreatic acinar cells [5,6]. We elucidated the glycosaminoglycan (GAG) binding properties of annexins IV, V and VI and showed that each of them has its own specific binding activities to GAGs [7]. Furthermore, we reported that annexin IV contributes to zymogen granule formation, presumably by interacting with GP-2, the most abundant glycoprotein in zymogen granule membranes (25–40% of membrane protein), functions as a critical component in the secretory granule formation and in the regulated membrane trafficking along the apical secretory pathway [6].

Preliminary immunohistochemistry experiments of bovine pancreas revealed that annexin VI is localized in the ducts, acinar cells nuclei and islet of Langerhans. This study suggested the possibility that annexin VI plays a role in the pancreatic endocrine secretory pathway. The roles of various annexins had already been investigated in exocytosis. In adrenal chromaffin cells, annexin II [8,9] and VII [10] are required in the secretory machinery. In pancreatic β cells, annexin I [11-14], II [11], VII [15], XI [16] were suggested to play a role in the regulation of the release of secretory vesicles. However, the mechanisms by which annexins regulate on the secretory machinery have not yet been fully explained.

In this study, we examined the function of annexin VI and GAGs in pancreatic β cells. We investigated GAGs as potential endogenous ligands of annexin VI in β cell line lysate, and we demonstrated the effect of annexin VI on insulin secretion with a membrane permeabilization technique using streptolysin-*O* (STLO). The possible role of annexin VI in the endocrine pancreas is also discussed.

2. Materials and Methods

2. 1. Materials

Rat pancreases were excised from freshly killed rats purchased from Japan Biological Materials Center (Tokyo, Japan). HIT-T15 cells were from ATCC (Manassas, USA). MIN6 cells were kindly donated by Prof. J. -I. Miyazaki (Osaka University). Roswell park memorial institute (RPMI) 1640 and Dulbecco's modified Eagle medium (DMEM) were from IWAKI (Funabashi, Japan). Fetal bovine serum (FBS) was from JRH BIOSCIENCES (Lenexa, USA). BCA Protein Assay Reagent was from PIERCE (Rockford, USA). Mouse monoclonal antibodies against insulin were from Biogenesis (Poole, UK). Rabbit polyclonal antibodies to annexin IV, V and VI were prepared according to the method reported by Harlow *et al* [17]. Horseradish

peroxidase (HRP) -conjugated goat anti-rabbit IgG antibodies, anti-mouse IgG and anti-mouse IgM were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, USA). Mouse HepSS-1 monoclonal antibodies, chicken CS-56 monoclonal antibody, human F69-3G10 monoclonal antibodies, Heparitinase II, heparan sulfate (bovine kidney), chondroitin sulfate A (whale cartilage) and B (pig skin) were from Seikagaku Kogyo (Tokyo, Japan). Streptavidin-biotinylated horseradish peroxidase complex (HRP-ABC) was from MP Biomedicals (Irvine, USA). Fluorescein isothiocyanate (FITC) -conjugated anti-rabbit IgG, biotinylated Texas Red anti-mouse IgM and heparin (porcine intestinal mucosa) were from Wako Pure Chemicals (Osaka, Japan). 48-MultiWell plate was from Sumitomo (Tokyo, Japan). STLO was from Sigma (St. Louis, MO, USA). Recombinant proteins were produced as a GST fusion protein as described previously [6,18]. The insulin ELISA kit was from Morinaga (Yokohama, Japan).

2. 2. Cell culture

Rat insulinoma cell line HIT-T15 cells were cultured in RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2.0 g/l sodium bicarbonate, and 10% FBS at 37°C in a humidified atmosphere of 95% air/5% CO_2 [19,20]. Mouse insulinoma cell line MIN6 cells were cultured in DMEM supplemented with 75 mg/l penicillin, 50 mg/l streptomycin and 15% FBS at 37°C in a humidified atmosphere of 95% air/5% CO_2 [21]. The culture medium was changed every 3-4 days. When the cells were 80-90% confluent, they were harvested with trypsin/EDTA.

2. 3. Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded rat pancreas were deparaffinized with xylene and then rehydrated with a graded series of ethanol (100-70%). The sections were rinsed with distilled water, washed with phosphate-buffered saline (PBS), then blocked by incubation with a mixture of 3% bovine serum albumin (BSA), 10% normal horse serum and 10% normal goat serum in PBS. They were incubated with a mixture of anti-annexin antibodies and anti-insulin antibodies overnight then washed in PBS. After incubation with the secondary antibodies and washed in PBS, they were incubated with avidin-conjugated Texas Red then washed with PBS. The sections were fixed with Mowiol 4-88 [22] and examined with a confocal laser scanning microscope TCS NT (Leica).

After washing with PBS, HIT-T15 cells and MIN6 cells grown on the cover glass were fixed with 4% paraformaldehyde in PBS and treated with 0.5% Triton-X 100 in PBS. The cells were immersed in 10% normal goat serum in PBS and 5% BSA in PBS. They were then incubated overnight with a mixture of primary antibodies and 0.5% Triton-X 100 in PBS. After washing with PBS, the cells were incubated with secondary

antibodies and 0.5% Triton-X 100 in PBS for 1 h and fixed with PBS and examined.

2. 4. Preparation of cell lysates

At 80% confluency, the cells were washed with PBS and then frozen at -80°C for 10 min. They were then solubilized with 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 1% NP-40 (PI buffer), 0.1% SDS, 0.5% sodium deoxycholate and 250 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were centrifuged for 20 min at 15,000 × *g* and the supernatants were collected. The protein concentrations of the lysates were determined with BCA Protein Assay Reagent.

2. 5. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis

One-dimensional SDS-PAGE was carried out by the method of Laemmli [23]. The electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and then probed with the primary antibodies. The bound antibodies were detected with HRP-conjugated anti-rabbit IgG or anti-mouse IgM and 4-choro-1-naphtol or chemiluminescence [5,7,18].

Alkaline treatment of Western blot membrane was carried out by incubation in 0.25 mM NaOH overnight at room temperature. The membrane was then washed with 0.25 mM acetic acid, and subjected to the immunostain analysis.

2. 6. Affinity adsorption

All procedures were performed at 4°C. One hundred μl of Glutathione (GSH)-Sepharose 4B gel (Pharmacia) were washed with 1 ml Tris-buffered saline (TBS) (pH 7.6) and centrifuged for 5 min at 1000 × *g*. The gel was then incubated in the TBS solution containing 0.5 mg GST, GST-annexin IV or GST-annexin VI overnight. After centrifugation for 5 min at 1000 × *g*, the precipitate was GST-GSH-Sepharose or GST-annexin-GSH-Sepharose gel. These propitiates were washed with 1 ml TBS (pH 7.6) containing 5 mM $CaCl_2$, and centrifuged for 5 min at 1000 × *g*. The cell lysate containing 1 mg protein and supplemented with 5 mM $CaCl_2$ was incubated with a 100 μl GST-GSH-Sepharose gel overnight. The supernatant obtained by centrifugation for 5 min at 1000 × *g* was then incubated 100 μl GST-annexin-GSH-Sepharose gel overnight. After washing with TBS, the gel was subjected to SDS-PAGE followed by immunoblot analysis.

2. 7. Insulin secretion from permeabilized MIN6 cells

Insulin secretion of MIN6 cells was performed by the method of Iino *et al* [16]. MIN6 cells were seeded at a density of 2 × 10^4 cells per well into a 48-MultiWell 3 days prior to an experiment. On the day of

experimentation, MIN6 cells were preincubated for 1 h at 37°C in 0.5 ml of HEPES-Krebs buffer (20 mM HEPES, 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.0 mM $NaHCO_3$, pH 7.4) supplemented with 5 mg/ml BSA. After washing twice with 0.5 ml of the glutamate buffer containing 100 mM K-glutamate, 42 mM Na-glutamate, 16 mM HEPES (pH 7.0), 3 mM MgATP, 1 mM EGTA and 5 mg/ml BSA with 10 nM Ca^{2+}, the cells were further incubated for 30 min in 0.5 ml of the glutamate buffer with 250 U/l STLO and various substances. $CaCl_2$ was added to the glutamate buffer to give an arbitrary concentration of free Ca^{2+}. At the end of the 30 min incubation, the medium was spun briefly to sediment any detached cells, and the supernatant was retained for insulin assay. Insulin released into the media was assayed by Insulin ELISA Kit.

3. Results

3. 1. Localization of annexin VI in endocrine pancreas

Formalin-fixed and paraffin-embedded sections of normal rat pancreas and formalin-fixed HIT-T15 cells derived from rat were stained immunohistochemically using anti-annexin VI and anti-insulin antibodies (Fig. 1). Immunostaining for insulin (red color) was found to be localization of β cells (Fig. 1B, E). Annexin VI was localized in pancreatic islets including β cells (Fig. 1C) and HIT-T-15 cells (Fig. 1F). As shown in Fig. 1C, the cytoplasm was mostly positive for annexin VI immunoreactively, but the nuclei were negative. Annexin IV and V were also demonstrated to be present in rat pancreatic islets and HIT-T15 cells (data not shown).

3. 2. Detection of annexin VI and GAGs in cultured cells

The Western blot analysis showed that cell lysates from HIT-T15 cells and MIN6 cells contain annexin VI with a molecular mass of 66 kDa (Fig. 2A). Annexin IV and V were also demonstrated to be present in these lysates (data not shown).

The lysates were also shown to contain a broad band with *ca.* 200 kDa positive to HepSS-1 monoclonal antibodies, specific to heparan sulfate (Fig. 2B). The band around 70 kDa was suggested to be BSA from cell culture medium that is reactive with this antibody.

When MIN6 cells lysate treated with Heparitinase II, was analyzed using F69-3G10, monoclonal antibodies to the heparan sulfate neo-epitope, generated by digesting heparan sulfate with heparitinase from Flovobacterium heparinum, the protein bands of *ca.* 70, 55, 30 kDa were detected on the membrane (Fig. 2C). These results indicated that the cells contain heparan sulfate proteoglycans. However, no chondroitin sulfate proteoglycans bands were detected in these cell lysates when CS-56 monoclonal antibodies were used (data not shown).

3. .3 Binding of GAGs in culture cell derived from β cells to annexin VI-Sepharose gel

Affinity adsorption technique was used to find ligands of annexin VI. Although each of annexins has its own specific binding activities to GAGs [7], annexins directly immobilized on Sepharose loose their binding activities. In this study, GST fusion annexins immobilized on GSH Sepharose were used as an adsorbent (Fig 3A).

After washing, the gel was subjected to SDS-PAGE, electroblotted onto a PVDF membrane and allowed to react with HepSS-1 monoclonal antibodies. The result showed a broad positive band with *ca.* 200 kDa (Fig. 3B). When the lysate was treated with alkali, the positive band was not detected anymore. The results indicated that HIT-T15 cells have heparan sulfate proteoglycans, which can be a ligand of annexin VI. No heparan sulfate proteoglycans bands were detected by the similar experiment using an annexin IV adsorbent (data not shown).

3. 4. Insulin secretion from permeabilized MIN6 cells

Annexin VI has a unique structure with eight conserved repeating units, in contrast to all other annexins, which have only four repeating units. Annexin VI has two isoforms that differ with respect to a 6-amino acid insertion at the start of repeat 7 [24]. To investigate the structures of annexin VI responsible of insulin secretion, we prepared annexin VI mutants (Fig. 4A). Fig. 4B shows the insulin secretion obtained with annexin VI mutants permeabilized with 250 U/l STLO with 10 μM Ca^{2+} glutamate buffer. Addition of full-length annexin VI (WT) lead to an increased insulin release from permeabilized MIN6 cells, while all mutants examined had almost no effect on insulin secretion.

We also investigated the effects of GAGs on insulin release from permeabilized MIN6 cells. Annexin VI bound to heparan sulfate and heparin in a calcium-independent manner, and chondroitin sulfate A and B in a calcium-dependent manner [7]. Fig. 5 shows that all GAGs inhibited insulin release in the presence of 10 μM Ca^{2+}. In permeabilized MIN6 cells, a significant increase of insulin release was observed when Ca^{2+} was added at concentrations higher than 10 nM [16]. Thus, GAGs used in this study inhibited Ca^{2+}-induced insulin release from permeabilized MIN6 cells.

4. Discussion

Annexin I [11,12], II [11], VII [15] and XI [16] are present in pancreatic β cells and have been suggested to play a role in the regulation of the release of secretory vesicles in β cells. We could detect annexin

VI in β cells (Fig. 1 and 2A) although Thomas *et al* [25] did not observe annexin VI in pancreatic endocrine cells and was also detected in the ducts as previously reported [25]. We could also identify annexin IV and V in β cells and the ducts (data not shown). Immunofluorescence localizations of annexin IV, V and VI were wider than that of insulin, β cells localized in that are arranged in the center of pancreatic islets. Since insulin immunoreactive regions correspond to β cells, annexin IV, V and VI are therefore present in not only β cells but also in α and δ cells. In conclusion we suggest that annexin VI has a role in both pancreatic exocrine and endocrine functions.

We elucidated the GAG binding properties of annexins IV, V and VI and showed that each of them has its own specific binding activities [7]. Annexins have various functions [4], presumably linked to their binding properties: sugar chains-sugar recognized proteins. In this study, immunoblot assay detected heparan sulfate proteoglycan in insulinoma cell lines lysates (Fig. 2B, C) but not chondroitin sulfate proteoglycan (data not shown). Furthermore, affinity adsorption assay showed annexin VI bound to the heparan sulfate proteoglycan derived from HIT-T15 cells (Fig. 3B). These results suggest that heparan sulfate proteoglycan is the endogenous ligand of annexin VI in β cells. Converting heparan sulfate proteoglycan in β cells, perlecan is synthesized and secreted [26]. On SDS-PAGE, perlecan has an apparent molecular weight > 450 kDa [27], but in our study, the band of heparan sulfate proteoglycan was detected at molecular weight of *ca*. 200 kDa, which is obviously different from those of perlecan. It could not be detected in HIT-T15 cells, under our experimental conditions. From β-TC3 cells, mouse insulinoma cell line, [S^{35}]sulfated-labeled proteoglycans eluted from diethylaminoethyl Sephacel at 0.35 M NaCl. Chromatography on Sepharose CL-4B and SDS-PAGE analysis revealed heparan sulfate proteoglycan of 450 kDa (perlecan) and of 200 kDa. Heparan sulfate proteoglycan of 200 kDa were present in the cell and in the medium. Thus, β-TC3 cells synthesize and secrete heparan sulfate proteoglycan of 200 kDa sensitive to Heparinase I and II digestion [28]. Although this heparan sulfate proteoglycan has not been fully characterized, it may be identical to those detected in HIT-T15 cells (in our study) and β-TC3 cells [28]. Heparan sulfate proteoglycans, including perlecan, have been found in amyloid deposits in the pancreatic islets of individuals with type II diabetes [29]. However, on the other hand heparan sulfate proteoglycan of 200 kDa is present both in the cell and medium as mentioned earlier. Although the amino acid sequences of the heparan sulfate proteoglycan are unknown, it may have a membrane spanning domain as syndecan. The role of the heparan sulfate proteoglycan in pancreatic endocrine systems remains to be investigated. In pancreatic exocrine cells, we previously reported that GP-2/proteoglycan submembranous matrix may play a role in membrane sorting during granule assembly in the trans-Golgi networks and in trafficking of ZG membranes from the apical plasma membrane after exocytosis. Recently we found that proteoglycans with molecular masses higher than 200 kDa are present in the peripheral components of the zymogen granule membranes. Because annexin IV interacts with various glycosaminoglycan chains in the presence of calcium [7],

annexin IV may play a role in the cross-linking between GP-2 and proteoglycans [6]. In the pituitary gland, sulfated proteoglycans do not play a role in the sorting or storage of regulated secretory proteins because glycosaminoglycans are rapidly secreted by the constitutive secretory pathway [30]. However, this is a story in pituitary, the story in pancreatic endocrine system is unknown. Annexin VI is not only present in the cytoplasm but also on the cell surface[31]. It is therefore suggested that annexin VI on β cell surface and heparan sulfate proteoglycan synthesized and secreted by β cells could interact each other in the cell-extracellular matrix, but further studies are necessary.

Annexin I, II, IV, VI and VII mediate membrane vesicle aggregation [4]. Particularly, annexin II and VI have been found in the plasma membrane of exocytic and secretory vesicles and have directly been implicated in the regulation of different steps of endo and/or exocytic trafficking pathways. It has been demonstrated *in vitro*, that annexin II promotes aggregation of chromaffin granules of endocrine cells [8]. The binding site of annexin VI has been mapped to the unique linker region connecting the two four-repeat lobes of protein. This region is not found in other annexins, thus emphasizing the bivalency of the interaction. The duplication of the core domain generates a second calcium-dependent phospholipid-binding module, thus allowing for two spatially separated membrane interactions [32]. The association of cholesterol and phosphatidylcholine in cholesterol-rich membrane microdomains induces the formation of phosphatidylserine-rich domains in directly neighboring membrane microdomains, which stimulate, the binding of annexin VI to phosphatidylserine [33]. Furthermore, cholesterol- and glycosphingolipid-rich microdomains (lipid raft) are present within the membrane of secretory granules of β cells [34,35]. And it is known that annexin VI associates with raft fractions from synaptic plasma membranes in a calcium-dependent manner [36]. In this study, insulin secretion assay from permeabilized MIN6 cells showed that WT promotes insulin release (Fig. 4B). Since STLO permealize the plasma membrane but not vesicle membrane, it seems that annexins play a role outside the granule, but not in the granule. This result suggests the possibility that annexin VI is involved in the regulation of insulin release by binding to secretory vesicles membrane. Inhibition of Ca^{2+}-dependent insulin release by GAGs (Fig. 5) suggested that GAGs blocked binding of annexin VI to membrane vesicle. The actual localizations of GAGs in β cells are not clear. The decrease of annexin-enhanced insulin secretion by GAG may be the result of the inhibition of annexin binding to the secretion granules. In addition, WT promotes insulin release, but not other mutants (Fig. 4B). Both WT and the alternative splicing form (ALT) exist *in vivo* [24]. *In vitro*, all annexin VI mutants bound to phosphatidylserine in calcium-dependent manner and eight-repeating forms (WT and ALT) bound more strongly than half fragments (our unpublished results). In addition, mutants without the splicing domain (ALT and C-) bound to GAGs more strongly than other mutants (our unpublished results). Since WT is the only molecular species to increase the insulin release, probably due to its specific tertiary structure. The

structure of WT in crystals was known [37], however other mutants was not.

Although annexin VI was referred that it did not observe in pancreatic endocrine cells [25], annexin VI was suggested to exit in both pancreatic exocrine and endocrine cells, and to interact with both cell-extracellular matrix heparan sulfate proteoglycan and membrane vesicle in β cells. As annexin VI is involved in many functions including insulin secretion process in β cells, the function of annexin VI in β cells is now under investigation. The analysis of the core protein of heparan sulfate proteoglycan of 200 kDa may also help the elucidation of the roles of annexin in β cells.

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Figure legends

Fig. 1. **Distribution of annexin VI and insulin in rat pancreatic islets and HIT-T15 cells.**

Three μm thick-sections of the rat pancreas (A, B and C) or HIT-T15 cells (D, E and F) were double-stained with anti-annexin VI (A and D), and anti-insulin (B and E) antibodies, then examined with confocal laser scanning microscopy. Annexin VI immunopositive cytoplasm was mostly positive for insulin immunoreactively and shown in yellow in double-stained images (C and F). The ducts (d) were stained with anti-annexin VI antibodies. Bars = 10 μm.

Fig. 2. **Detection of annexin VI and heparan sulfate proteoglycan.**

A, Detection of annexin VI. HIT-T15 cell (lane 1) and MIN6 cell (lane 2) lysates were analyzed by SDS-PAGE on 9.5% acrylamide gel followed by electroblotting to a membrane. The proteins on the membrane were subjected to immunostaining with polyclonal antibodies against annexin VI. B, Detection of heparan sulfate proteoglycan. HIT-T15 cell (lane 1) and MIN6 cell (lane 2) lysates were analyzed by SDS-PAGE on 7.5% acrylamide gel followed by electroblotting to a membrane. The proteins on the membrane were subjected to immunostaining with HepSS-1 monoclonal antibodies, specific to heparan sulfate. kDa = kilodalton. C, Detection of heparan sulfate proteoglycan after heparitinase treatment. MIN6 cell lysate without (lane 1) or with Heparitinase II treatment (lane 2) was analyzed by SDS-PAGE followed by electroblotting to a membrane. The proteins on the membrane were subjected to immunostaining with F69-3G10 monoclonal antibodies reacted with Δheparan sulfate.

Fig. 3. **Detection of glycoconjugates by gel adsorption.**

A, Structure of GST-annexin-GSH Sepharose gel. B, SDS-PAGE and immunostaining of GST-annexin VI-GSH Sepharose gel complex. The complex was directly analyzed by SDS-PAGE followed by electroblotting to a

membrane. The proteins on the membrane were subjected to immunostaining with HepSS-1. Lane 1, treated without alkali; lane 2, treated with alkali.

Fig. 4. **Effect of annexin VI and deletion mutants on Ca^{2+}-induced insulin release from STLO-treated MIN6 cells.**

A, Schematic representation of annexin VI and the deletion mutants structures [31]. WT, full-length human annexin VI; ALT, the alternative splicing form; N, the N-terminal annexin VI specific domain and the N-terminal half of annexin VI-core region; C+, the N-terminal annexin VI specific domain and the C-terminal half of the annexin VI-core region; C-, the N-terminal annexin VI specific domain and the C-terminal half without the alternative splicing domain. B, After 1 h preincubation, MIN6 cells (2×10^4 cells/well) were permeabilized with 250 U/l STLO and incubated for 30 min with 10 μM Ca^{2+} glutamate buffer in the presence or absence of 1 μg/ml recombinant annexin VI.

Fig. 5. **Effect of glycosaminoglycans on Ca^{2+}-induced insulin release from STLO-treated MIN6 cells.**

After 1 h preincubation, MIN6 cells (2×10^4 cells/well) were permeabilized with 250 U/l STLO and incubated for 30 min with Ca^{2+} glutamate buffer at different concentrations of Ca^{2+} in the presence of heparan sulfate (open squares), heparin (open circles), chondroitin sulfate A (open triangles), chondroitin sulfate B (crosses) or in the absence of GAGs (closed diamonds). The concentrations of GAGs were 2 μg/ml respectively.

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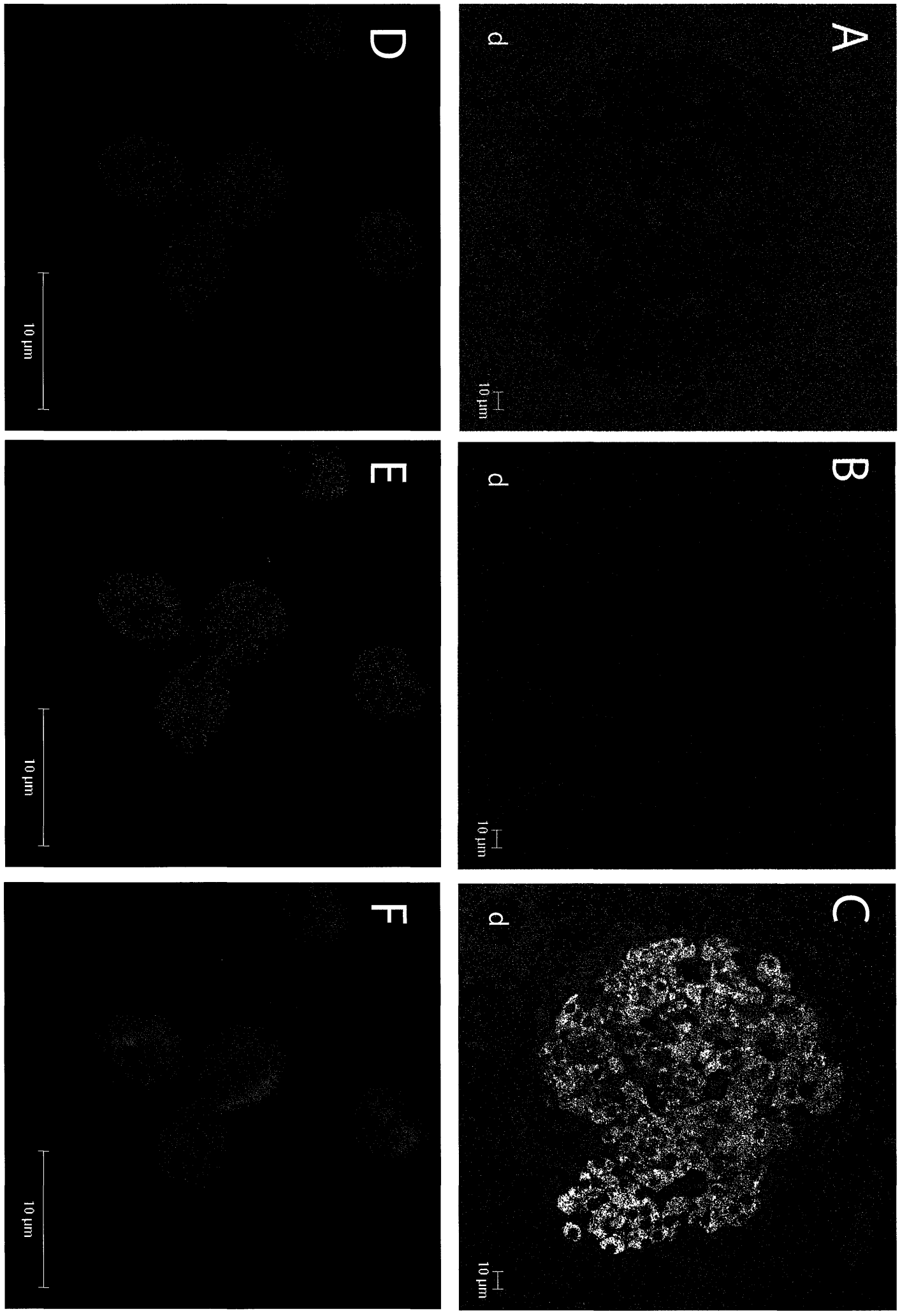

Figure 1

Figure 2

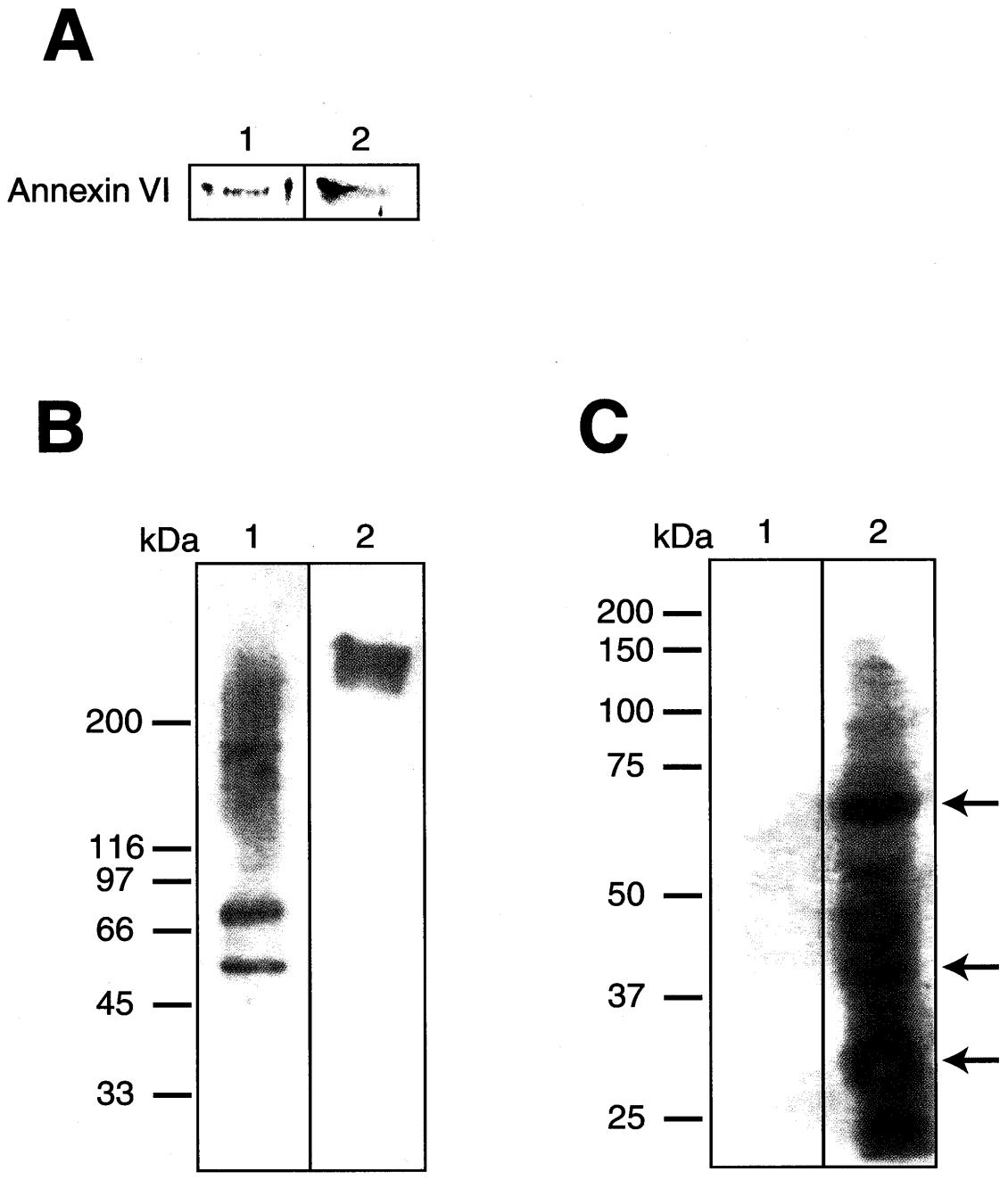
A
1
2
Annexin VI
B
kDa
1
2
200
116
97
66
45
33
C
kDa
1
2
200
150
100
75
50
37
25

Figure 3

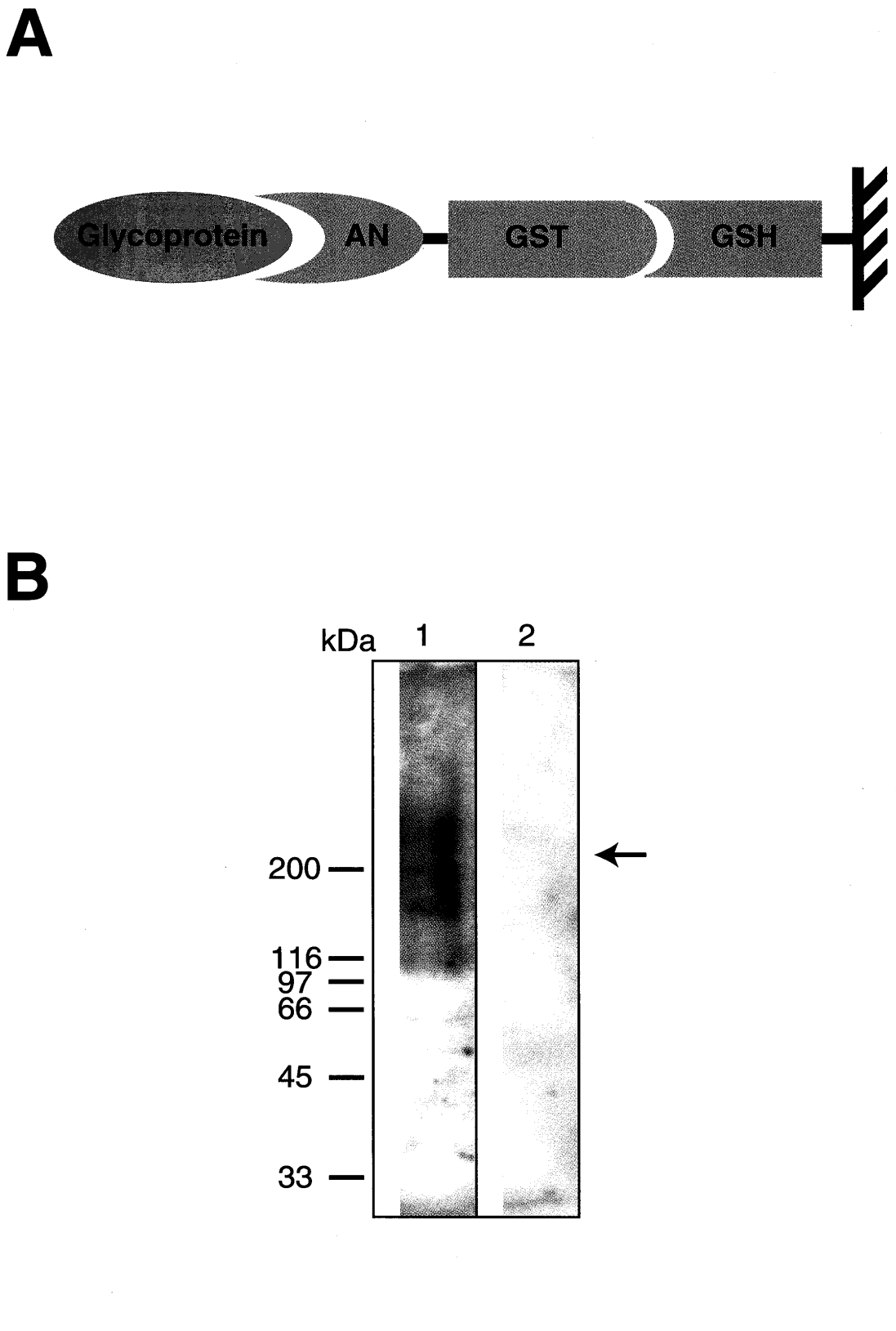

Figure 4

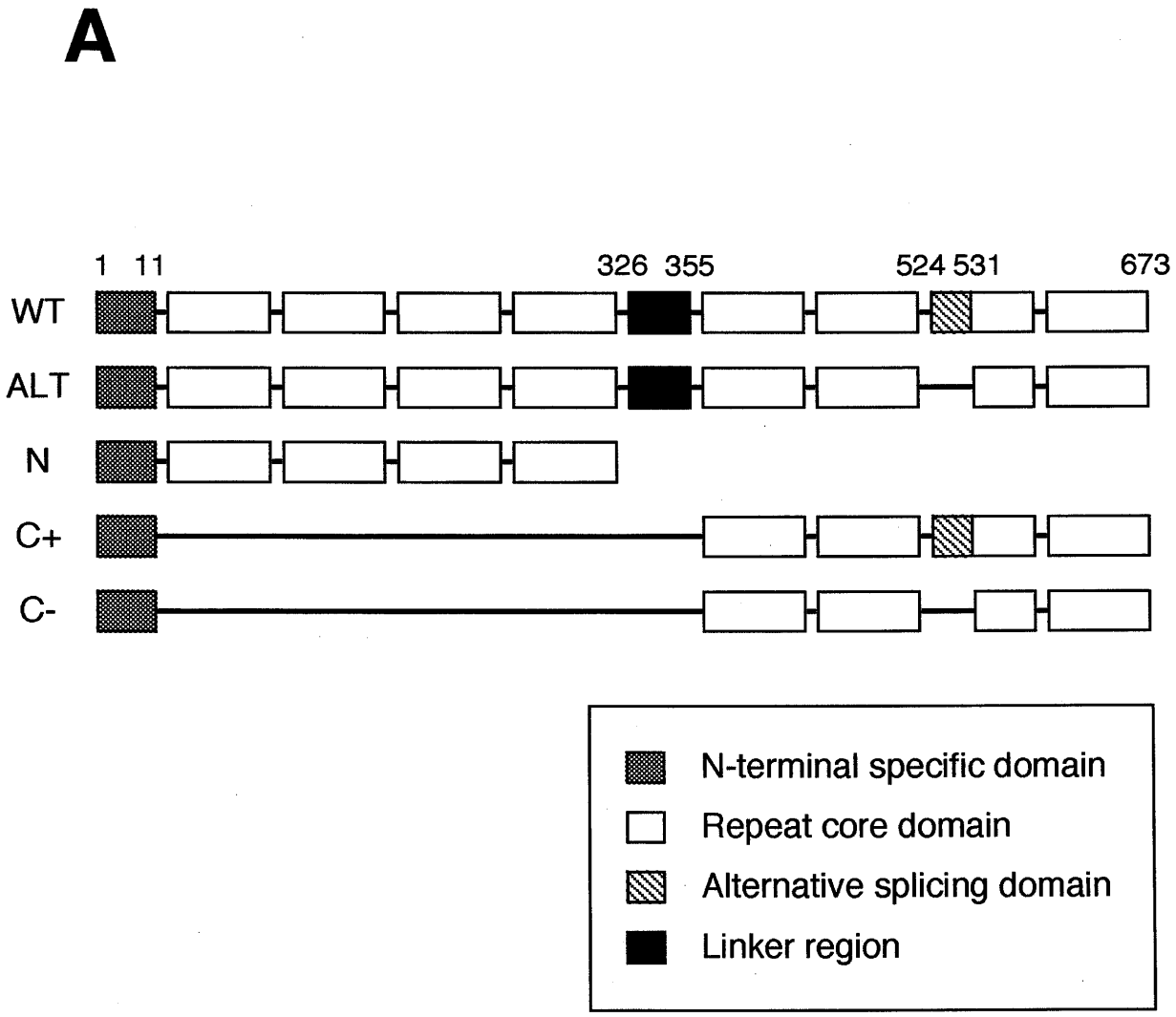
A
1 11
326 355
524 531
673
WT
ALT
N
C+
C-
N-terminal specific domain
Repeat core domain
Alternative splicing domain
Linker region

Figure 4

B

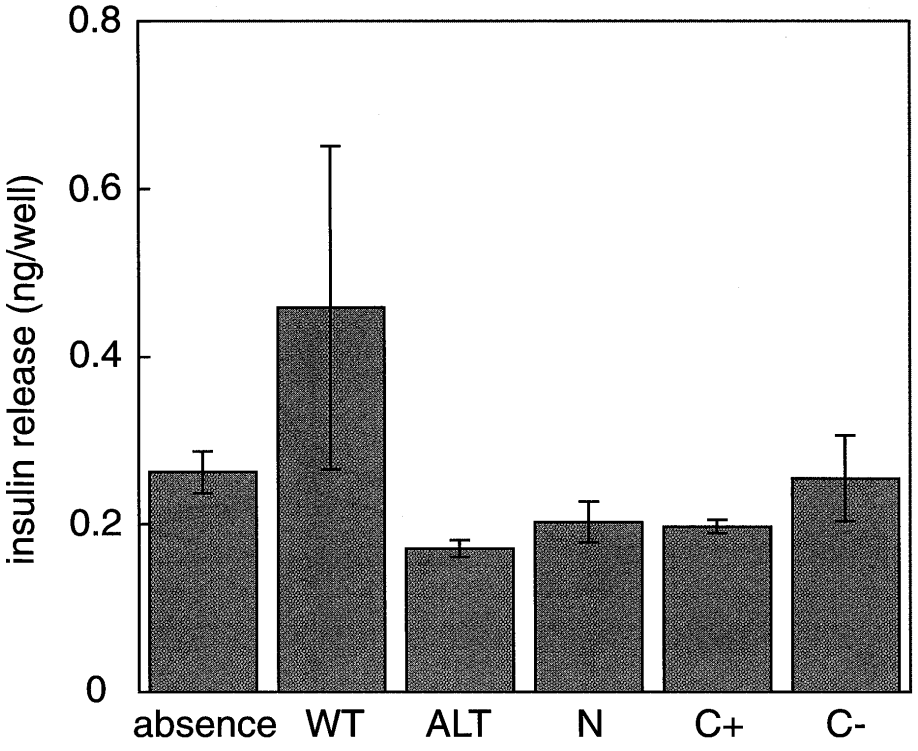

Figure 5