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(54) Title: PHARMACEUTICAL COMPOSITION FOR INCREASING INSULIN SECRETION FROM PANCREATIC  $\beta$ -CELLS

(57) Abstract: The present invention relates to a pharmaceutical composition comprising (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or a mimetic of the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN; (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin; (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95; (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95; (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.

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## **Pharmaceutical composition for increasing insulin secretion from pancreatic $\beta$ -cells**

The present invention relates to a pharmaceutical composition comprising (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or a mimetic of the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN; (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin; (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95; (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95; (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110. Further, the present invention relates to the use of (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or of a mimetic of the C-terminus of ICA512, pro-

ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN; (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, wherein the C-terminal fragment removed includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin; (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95; (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95; (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110 for the preparation of a pharmaceutical composition for the treatment of diabetes.

Several documents are cited throughout the text of this specification. The disclosure content of the documents cited herein (including manufacturer's specifications, instructions, etc.) is herewith incorporated by reference.

Diabetes mellitus is the most common metabolic disorder and its incidence is alarmingly increasing worldwide (Diamond, 2003; Zimmet et al., 2001). Autoimmune destruction of insulin producing  $\beta$ -cells causes type 1 diabetes (Castano and Eisenbarth, 1990; Tisch and McDevitt, 1996), whereas impaired insulin secretion in face of ever-increasing food intake and reduced physical activity leads to type 2 diabetes (Saltiel, 2001). Restoration of  $\beta$ -cell mass and function are therefore major goals in diabetes research.

Each  $\beta$ -cell stores  $\sim 10^4$  insulin secretory granules (Bratanova-Tochkova et al., 2002; Rorsman and Renstrom, 2003). Less than 5% of them are readily releasable, i.e. undergo exocytosis during the first phase insulin secretion, which lasts up to 10 minutes after the elevation of blood glucose  $>5$  mM. The remaining  $>95\%$  of the granules belong to the reserve pool. Recruitment of these granules for exocytosis accounts for the second phase of insulin secretion and requires their priming. This

consists of several ATP-,  $\text{Ca}^{2+}$ - and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P<sub>2</sub>]-dependent steps, which allow secretory vesicles to dock at the plasma membrane and enter the readily releasable pool (Klenchin and Martin, 2000). A critical step in this process is the mobilization of reserve granules. The motor protein kinesin is responsible for the ATP-dependent, microtubule-mediated transport of granules toward the cell surface (Donelan et al., 2002; Varadi et al., 2002), while myosin Va, also an ATPase, drives the movement of insulin granules on cortical actin microfilaments (Ivarsson et al., 2005; Varadi et al., 2005). By sustaining the ATP-dependent refilling of the readily releasable pool, fuel secretagogues such as glucose can greatly amplify insulin secretion relative to agents such as sulphonylureas, which evoke mostly, albeit not exclusively (Eliasson et al., 2003), the exocytosis of pre-existing primed granules by inducing membrane depolarization and  $\text{Ca}^{2+}$ -entry. The dense meshwork of cortical actin filaments, on the other hand, is thought to inhibit insulin secretion by restricting granule mobility and their access to the plasma membrane (Bruun et al., 2000; Li et al., 1994; Tomas et al., 2006). Despite recent progress (Waselle et al., 2003), however, little is known about the dynamic interaction of granules with the actin cytoskeleton. Knowledge of these mechanisms could be exploited to develop novel antidiabetic drugs.

It was recently proposed that islet cell autoantigen 512/IA-2 (ICA512) tethers insulin granules to actin microfilaments (Ort et al., 2000; Ort et al., 2001; Solimena and Gerdes, 2003). ICA512 is an evolutionary conserved member of receptor protein tyrosine phosphatase (PTP) family (Lan et al., 1994; Rabin et al., 1994; Zahn et al., 2001), which lacks phosphatase activity (Drake et al., 2003) and is mostly expressed in neuroendocrine cells (Solimena et al., 1996). The pro-form of ICA512 is a glycoprotein of 110 kDa, whose ectodomain is processed at a consensus cleavage site for furin-like convertases during the maturation of secretory granules (Solimena et al., 1996) (Fig. 7A). This cleavage originates a 65 kDa transmembrane fragment (ICA512-TMF) that in the case of human ICA512 includes amino acids 449-979 (Fig. 7A). The cytoplasmic domain of ICA512-TMF binds to the PDZ domain of  $\beta$ 2-syntrophin (Ort et al., 2000), which in turn interacts with cortical actin microfilaments through its binding to utrophin (Albrecht and Froehner, 2002). Glucose stimulation, on the other hand, may induce the dissociation of ICA512-TMF from  $\beta$ 2-syntrophin by

affecting the phosphorylation of the latter, hence increasing granule mobility (Ort et al., 2001).

Ca<sup>2+</sup>-dependent exocytosis of secretory granules triggers the transient insertion of ICA512-TMF into the plasma membrane and its intracellular cleavage by the Ca<sup>2+</sup>-activated protease calpain-1 (Ort et al., 2001). The resulting cytosolic fragment of ICA512 (ICA512-CCF, amino acids 659-979; Fig. 7A) contains the inactive PTP module flanked by the two PDZ domain interacting regions and enhances granule biogenesis by acting as a decoy phosphatase. Specifically, ICA512-CCF is targeted to the nucleus (Trajkovski et al., 2004) where it binds to tyrosine phosphorylated signal transducer and activator of transcription (STAT)-5 and -3 (Mziaut et al., 2006), thereby preventing their dephosphorylation by conventional tyrosine phosphatases and enhancing their transcription of insulin and other granule genes. Consistent with ICA512 having a role in granule biogenesis and trafficking, knockout of its gene in mice leads to reduced insulin secretion and glucose intolerance (Saeki et al., 2002), whereas its overexpression in mouse insulinoma MIN6 cells increases the number of granules, the content and half-life of insulin as well as its stimulated secretion (Harashima et al., 2005). Valuable approaches to treat diabetes have been described in WO2006/029728 A1. This patent application has for the first time linked that C-terminal fragments of ICA512 are targeted to the nucleus of pancreatic  $\beta$ -cells and stimulate the expression of peptide hormones such as insulin. Due to the ever increasing number of patients suffering from diabetes, it would, however, be valuable to develop alternative approaches to medicaments useful in the treatment of diabetes.

Therefore, the technical problem underlying the present invention the provision of additional compounds useful as ingredients or in the development of medicaments for the treatment of diabetes.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a pharmaceutical composition comprising

- (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or a mimetic of the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;
- (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
- (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
- (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.

In accordance with the present invention, the term "pharmaceutical composition" relates to a composition for administration to a patient, preferably a human patient. The pharmaceutical composition of the invention comprises the compounds recited above, alone or in combination. It may, optionally, comprise further molecules capable of altering the characteristics of the compounds of the invention thereby, for example, stabilizing, modulating and/or activating their function. The composition may be in solid, liquid or gaseous form and may be, *inter alia*, in the form of (a) powder(s), (a) tablet(s), (a) solution(s) or (an) aerosol(s). The pharmaceutical composition of the present invention may, optionally and additionally, comprise a

pharmaceutically acceptable carrier. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions, organic solvents including DMSO etc. Compositions comprising such carriers can be formulated by well known conventional methods. The term "parenteral" as used herein refers to modes of administration, which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. These pharmaceutical compositions can be administered to the subject at a suitable dose. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. The therapeutically effective amount for a given situation will readily be determined by routine experimentation and is within the skills and judgement of the ordinary clinician or physician. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1  $\mu$ g to 5 g units per day. However, a more preferred dosage might be in the range of 0.01 mg to 100 mg, even more preferably 0.01 mg to 50 mg and most preferably 0.01 mg to 10 mg per day.

Additionally, a nucleic acid molecule and preferably a DNA molecule encoding said compound(s) (wherein the term "compound" also refers to the mentioned proteins as well as the fragments or derivatives or modifications thereof) may be formulated into a pharmaceutical composition. The nucleic acid molecule is eventually to be introduced into the desired cells. Appropriate formulations include those wherein  $10^6$  to  $10^{12}$  copies of the DNA molecule, advantageously comprised in an appropriate vector are administered per dose. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host/cells.

In accordance with this specification, "ICA512" refers to the mature transmembrane protein "Islet Cell Autoantigen 512" also referred to as "IA-2" or "protein tyrosine phosphatase N" (PTPN) having a level of identity of at least 30%, preferably at least about 35%, more preferred at least 50% and most preferred at least 75% such as 85% with the amino acid sequence displayed in SEQ ID NO.: 1 which reflects the human ICA512 sequence. This sequence is also available as entry # Q16849 from the NCBI protein database. This amino acid sequence, in accordance with the invention, is preferably encoded by the nucleotide sequence of SEQ ID NO.: 2 which is available as entry # L18983 from the NCBI nucleotide database. It is to be understood that the various ICA512 proteins having different degrees of identity and subsumed under the term "ICA512" solely denote naturally occurring proteins from other species that may, in the scientific literature, bear a name different from ICA512. Thus, a mouse ICA512 protein (NCBI entry #I48721) has been identified that bears 86.7% identity with the human sequence at the amino acid level. Similarly, *Rattus norvegicus* (rat) (NCBI entry #Q63259, 86.82% identity), *Danio rerio* (zebrafish) (NCBI partial sequence # AF190144.1, 75.97% identity), and *C. elegans* (NCBI entry # CAB52188, 38.10% identity) proteins have been identified. Further, the invention explicitly includes allelic variants from humans. All these proteins have the following functions in common: 1) they all include a single atypical protein tyrosine phosphatase (PTP) domain in their cytosolic tail that lacks phosphatase activity towards conventional tyrosine phosphatase substrates, 2) they are most abundant in neuroendocrine cells; 3) they are enriched in secretory granules (SGs). They may all be used in accordance with the method of the present invention.

The term "ICA512" also includes derivatives of ICA512 having ICA512 function in accordance with the present invention. The term "derivative of ICA512 having ICA512 function" relates to a protein (the term "protein" in connection with this specification having the same meaning as the term "polypeptide") that is derived from naturally occurring ICA512, e.g. by post-translational modifications that are effected by eukaryotic cells other than the cells the protein naturally occurs in or by lack of post-translational modifications (in case the protein is produced in prokaryotic organisms), by chemical modification, by peptidomimetics etc., to name a few important options. In all these options, the ICA512 function is retained. "ICA512 function" means, in

accordance with this invention, to include a PTP like-domain, the capability to be cleaved by  $\mu$ -calpain, also known as calpain-1, and to sequester a C-terminal portion that retains the capacity to be targeted to the nucleus, and/or to bind proteins of the PIAS (Protein Inhibitor of Activated STAT) family, and/or to instigate or enhance the expression of genes encoding peptide-hormones by modulating the activity of transcription factors, such as Signal Transducer and Activator of Transcription (STATs) as described in WO2006/029728. Included within the term "derivative" are also/thus molecules that have the above identified biological function and that are (semi)synthetically or recombinantly produced without assuming first the naturally occurring structure of ICA512 which is then subsequently modified, e.g. by post-translational modifications that are effected by eukaryotic cells other than the cells the protein naturally occurs in. This is the case if the derivative is a peptidomimetic. Alternatively, the term includes fragments of the full-length protein that are modified as outlined above and that retain the function specified above. Modifications referred to above (in particular post-translational and chemical) include: acetylation, acylation, amidation, ADP-ribosylation, glycosylation, GPI anchor formation, covalent attachment of a lipid or lipid derivative, methylation, myristylation, pegylation, prenylation, phosphorylation, ubiquitination and sumoylation. Derivatives of the above protein include further fusion proteins wherein the two fusion partner may, e.g., be fused via a (flexible) linker such as a glycine linker or a gly-ser linker or wherein the protein is fused to a tag such as His-tag, FLAG-tag, myc-tag etc.

The term "promoting the presence" in general means that a certain level of the protease or the serine/threonine kinase or the serine/threonine phosphatases, all as mentioned above, is maintained in the cells being the target of the pharmaceutical composition of the invention which are preferably pancreatic  $\beta$ -cells and most preferably human pancreatic  $\beta$ -cells. Accordingly, the invention envisages that, in one alternative, an existing level of any of the above recited enzymes in the cell is maintained or essentially maintained, such as maintained to at least 70%, preferably to at least 80%, more preferred to at least 90% and most preferred to at least 95% such as 100%. Important options to maintain an existing level of said enzymes are described in connection with preferred embodiments of the method of the invention further below. In addition, if the cell naturally does not produce any of said enzymes for example due to genetic defects, production may be stimulated by introducing a vector

capable of expressing at least one of said enzymes into said cell. Important options how the level of said compounds may be enhanced are discussed in connection with preferred embodiment further below. Further to the above, it is to be understood that the term "enhancing" in connection with the production of any of the enzymes as recited above relates alternatively or both to the enhancement of levels of any of said enzymes already existing in the cytoplasm or in the nucleus and to the *de novo* production thereof in a cell. The level of said compounds required in a cell that is necessary for stimulating expression may vary from cell type to cell type. In any case and as regards pancreatic  $\beta$ -cells, it is preferred that said cells are in a stimulated stage, i.e. secrete said insulin. In the case of pancreatic  $\beta$ -cells, stimulation may be effected by contact with or addition of glucose. It is particularly preferred that the promotion of the presence of either or all of said enzymes includes or corresponds to the promotion of the expression thereof. Also in the other embodiments described in accordance with this invention, "expression" is a preferred embodiment of "presence".

The term "expression" is well known in the art and relates to the transcription and/or translation of a gene, resulting in the generation of a messenger RNA and, optionally, subsequently, its translation into a protein. In accordance with the present invention, it is preferred that in particular the transcription of the gene is stimulated.

The term "promoting the [...] activity" refers, as was explained in conjunction with the term "promotion of the presence" to the essential maintenance or an increase of the activity. An increase of the activity may be affected, for example, by site-directed mutagenesis and the subsequent testing for an increased activity.

The term "ICA512-TMF" relates to the mature transmembrane form of ICA512. More specifically, ICA512 is released or generated from a precursor, pro-ICA512. Pro-ICA512 is a glycoprotein of 110 kD synthesized in a glucose-regulated fashion. Processing of its ectodomain along the secretory pathway by a furin-like protease converts pro-ICA512 into its mature transmembrane form of 65 kD (ICA512-TMF), which is enriched on secretory granules (SG).

The term "ICA512-CCF" refers to a cleavage product obtained from ICA512-TMF by the catalytic action of  $\mu$ -calpain. As mentioned above, this cytosolic fragment

contains the inactive PTP domain flanked by the two PDZ interaction regions. The activity of  $\mu$ -calpain has been described in detail in, for example, WO2006/029728.

The various derivatives and modifications of ICA512 described herein above and also encompassed by the present invention apply mutatis mutandis to ICA512-TMF and ICA512-CCF molecules.

The term "PHOGRIN", also known as IA-2beta or protein tyrosine phosphatase, receptor type, N polypeptide 2 (ptprn2), refers to a paralogue of ICA512 (Kawasaki et al, 1996; Chiang and Flanagan, 1996; Lu et al, 1996). Human PHOGRIN is encoded by SEQ ID NO: 6 and can be retrieved from the NCBI nucleotide database under the entry number NM\_002847. The amino acid sequence of human PHOGRIN corresponds to SEQ ID NO: 7 and can be retrieved from the NCBI protein database under the entry number AAC50472. Like ICA512, PHOGRIN lacks phosphatase activity, in particular toward phosphoproteins and is mostly expressed in neuroendocrine cells, including neurons and pancreatic  $\beta$ -cells. Comprised by the present invention are also derivatives and modifications of PHOGRIN wherein the derivatives and modifications of PHOGRIN are defined in an analogous manner with regard to the derivatives and modifications described for ICA512 herein above. The term "PHOGRIN" includes in addition molecules corresponding in sequence to ICA512-TMF and ICA512-CCF, in accordance with this invention.

The term " $\beta$ 2-syntrophin" refers to a protein having a level of identity of at least 30%, preferably at least about 35%, more preferred at least 50% and most preferred at least 75% such as 85% with the amino acid sequence displayed in SEQ ID NO.: 4 which reflects the human  $\beta$ 2-syntrophin sequence. This sequence is also available as entry Q13425 in the NCBI protein database, whereas e.g. the corresponding mouse sequence (SEQ ID NO: 5) has the entry number Q61235. The amino acid sequence, in accordance with the present invention, is preferably encoded by the nucleotide sequence of SEQ ID NO:3 which is available as entry NM\_006750 from the NCBI nucleotide database. It is to be understood that the various  $\beta$ 2-syntrophin proteins having different degrees of identity and subsumed under the term " $\beta$ 2-syntrophin" solely denote naturally occurring proteins from other species that may, in the scientific literature, bear a name different from  $\beta$ 2-

syntrophin.  $\beta$ 2-syntrophin is a cytosolic adaptor protein that interacts through its PDZ domain to a variety of receptor and signaling molecules and links them to the actin cytoskeleton via the association of its C-terminal domain to the dystrophin related protein utrophin (Albrecht and Froehner 2002). They may all be used in accordance with the method of the present invention.

The term "devoid of the C-terminus" of either of the above-recited molecules ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN relates to molecules wherein the C-terminus has been cleaved by proteases. The C-terminal fragment removed includes, at least in part, the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin of these molecules. Accordingly, also a portion of said functional domain can be cleaved off, wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin. In other terms, the term means that at least a part of the C-terminal PDZ-interacting domain of the above molecules is cleaved off or deleted resulting in a non-functional remaining portion of the C-terminal PDZ interacting domain or its complete absence. The C-terminal PDZ-interacting domain spans amino acids 951 to 979 of pro-ICA512 (SEQ ID NO: 1), i.e. it consists of 29 amino acids (see Figure 7A). It is present in ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN. It is preferred that domains of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN other than the C-terminal PDZ-interacting domain are not affected by said cleavage or deletion, i.e. that the cleavage or deletion of the C-terminal fragment does not exceed the C-terminal PDZ-interacting domain consisting of 29 amino acids or reach into the neighbouring PTP-like domain. It is important to note that this C-terminus is different from ICA512-CCF which is itself a C-terminal fragment of ICA512-TMF, ICA512 and pro-ICA512, and is obtained upon cleavage by the well-known enzyme  $\mu$ -calpain.

The term "sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin", means that at least a portion of the C-terminal PDZ-interacting domain of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN was cleaved off resulting in C-terminally deleted ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN fragments lacking a functional C-terminal PDZ-interacting domain. These C-terminally deleted fragments thus show a significantly weakened or preferably completely abrogated binding affinity to the PDZ domain of  $\beta$ 2-syntrophin and their binding to the PDZ domain of  $\beta$ 2-syntrophin is thus

hindered. Tests on how to assess whether the portion cleaved off is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin are well known to the skilled person and disclosed throughout this application. They include immunoprecipitation, pull-down assays as well as isothermal titration calorimetry (ITC) or native gel electrophoresis.

It has surprisingly been found in accordance with the present invention that various further approaches over those described in WO2006/029728 are available that allow the provision of compounds useful in the treatment of diabetes or useful in the development of lead compounds for the treatment of diabetes. The common inventive principle underlying all the approaches of the present invention is that all of the above-mentioned ingredients of the pharmaceutical composition of the invention directly induce the secretion of insulin from secretory granules without the *de novo* synthesis of insulin. More specifically, in accordance with the present invention, it could be demonstrated that the ICA512-TMF/ $\beta$ 2-syntrophin complex regulates the turnover of insulin granules by restraining their motility and exocytosis. In other terms, the experiments underlying the present invention surprisingly established that intracellular cleavage of ICA512-TMF, in addition to granule biogenesis, also regulates insulin secretion. As was further found in accordance with the invention, dissociation of the ICA512-TMF/ $\beta$ 2-syntrophin complex following granule exocytosis is further enhanced by the  $\text{Ca}^{2+}$ /calpain-1-induced generation of ICA512-CCF, which independently binds to ICA512-TMF and  $\beta$ 2-syntrophin. On the one hand, removal of the C-terminal PDZ binding motif is envisaged in accordance with the invention to further amplify insulin secretion by skewing the interaction of ICA512-CCF towards ICA512-TMF. C-terminal cleavage of each ICA512-TMF molecule in the granule membrane, on the other hand, is envisaged in accordance with the invention to progressively reduce the ability of a vesicle to anchor to the cortical actin cytoskeleton and thus its residence time near the plasma membrane. Such aging process could explain the preferential release of younger versus older granules (Duncan et al., 2003; Solimena and Gerdes, 2003), the latter being progressively displaced by the former from the cell cortex (Duncan et al., 2003). In other terms, following the dissociation of  $\beta$ 2-syntrophin from ICA512-TMF or ICA512-CCF the secretory granules move more freely and thus a contact with the cell membrane becomes more likely. Upon the contact of the secretory granules with the cell

membrane, a fusion of the secretory granules with the cell membrane becomes possible which results in the release of insulin contained in the secretory granules. Such a fusion will only occur if the cellular calcium channels are open which will in turn only be the case if the glucose concentration in the blood is enhanced. The various proteins or protein fragments mentioned above, all devoid of their C-terminus which is cleaved off by the novel protease do not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin. As a consequence the ICA512/ $\beta$ 2-syntrophin complex will not be formed or not be maintained with the consequence of an enhancement of insulin secretion. The mimetic of the C-terminus of ICA512 or of PHOGRIN or the mentioned cleavage products thereof function in a similar manner, namely by competing with ICA512/PHOGRIN for binding to  $\beta$ 2-syntrophin and thus not allowing the ICA512/ $\beta$ 2-syntrophin complex to form or driving it to dissociate.

In the course of the present invention, it has been found that ICA512-CCF with two functional PDZ interacting domains binds to  $\beta$ 2-syntrophin with higher affinity than C-terminally truncated ICA512 (deleted or cleaved) as assessed by immunoprecipitation (see Figure 4E). This may, at least in part, be due to the vicinity of both PDZ-interacting domains in the tertiary structure of ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN thus enabling for an enhanced interaction. The binding activity of the part of the PDZ-interacting domain remaining after cleavage or deletion to  $\beta$ 2-syntrophin can also be assessed using other approaches well known to the skilled person such as isothermal titration calorimetry (ITC) which is more quantitative technique that measures the binding activity ( $K_a$ ) between two molecules or more in solution.

In the context of the present invention, a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF denotes a protease the action of which results in an ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF fragment, which is devoid of a functional C-terminal PDZ-interacting domain as defined above.

Thus, the cleavage site could be located within the PDZ-interacting domain or at its N-terminus. Proteases cleaving the C-terminus of any of the above ICA512 or PHOGRIN molecules or fragments to obtain a fragment devoid of a functional C-

terminal PDZ-interacting domain can be determined by routine methods. For example, starting from the sequence of said C-terminus, proteases cleaving at a sequence contained within said C-terminus can be determined by routine computer-based prediction methods, available e.g. at <http://www.expasy.org/tools/peptidocutter/> or at scientific laboratories creating and publishing their own algorithms. Practical approaches to identify the protease that cleaves at the C-terminus in vivo as well as proteases in general that cleave the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN to obtain proteins with a non-functional PDZ-interacting domain are well-known to the skilled person. One approach includes the utilisation of various classes of proteases inhibitors in insulinoma cells expressing the cytoplasmic fragment of ICA512 fused at its N-terminal end to an HA tag (HA-ICA512-CCF). Western blotting of the electrophoretic profile of ICA512 that reveals absence of cleavage of ICA512-CCF will lead to the identification of at least the family to which the protease belongs and also the protease itself. Alternatively, commercially available arrays kit for various proteases can be used which provide a miniaturized platform that allows the screening of proteolytic activities genome wide. Incubation of recombinant HA-ICA512 with the arrayed proteases and analysis of the electrophoretic profile of HA-ICA512-CCF could be appropriate methods to reveal the identity of the protease. Another approach is the utilisation of an RNAi library to specifically knock down the proteases followed by Western blot analysis. The knock down showing the absence of the C-terminally cleaved ICA512-CCF is a clear indication of the identity of the protease.

The PDZ domain of  $\beta 2$ -syntrophin is flanked on both sides by a split pleckstrin homology domain, which in the case of  $\beta 2$ -syntrophin binds to PI(4,5)P<sub>2</sub>. Generation of PI(4,5)P<sub>2</sub> at the plasma membrane is a critical step for the priming and release of secretory granules (Gong et al., 2005; Hay et al., 1995). Binding of PI(4,5)P<sub>2</sub> to the pleckstrin-homology domain of  $\beta 2$ -syntrophin is envisaged in accordance with the present invention to affect the embedded PDZ domain, and thus represents an additional means to modulate the interaction of ICA512 and the granules with cortical actin.

Thus, whereas the applicant does not wish to be bound by any scientific theory, in accordance with the present invention, a model is proposed whereby intracellular cleavage of ICA512-TMF in proportion to granule exocytosis originates a short feedback loop that amplifies insulin secretion by increasing the mobility of granules, and hence their probability to encounter membrane sites competent for exocytosis (Fig. 6). Progressive depletion of granules, in turn, enhances the probability of ICA512-CCF to reach the cell nucleus, thereby promoting the transcription of insulin and other granule genes, and thus granule biogenesis. Further quantitative elaborations of these processes may allow the formulation of algorithms that illustrate how  $\beta$ -cells count granule stores and exocytotic events to dynamically regulate granule biogenesis and glucose homeostasis.

The above discussed experimental data and conclusions therefrom allow in accordance with the present invention to provide all the various novel ingredients of the pharmaceutical composition disclosed in accordance with the present invention. Specifically, since ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, all devoid of the C-terminus cleaved by the protease such that at least a portion of the PDZ binding/interaction domain is removed, lead to a increased insulin secretion from secretory granules, those compounds are of immediate interest as a pharmaceutical.

Similarly, the promotion of the presence, preferably expression, or of the activity of a protease that cleaves the C-terminus of any of the above-mentioned proteins will in a similar manner lead to a promotion of insulin secretion from secretory granules. In an alternative manner, the promotion of the presence or activity of a serine/threonine kinase phosphorylating  $\beta$ 2-synthrophin serine residue 95 will break up the interaction of  $\beta$ 2-synthrophin with ICA512-TMF or ICA512-CCF. -

An analogous result is achieved by interfering with the presence or activity of a serine/threonine phosphatase that dephosphorylates  $\beta$ 2-synthrophin on serine residue 95. A candidate phosphatase in the present invention would be Calcineurin. Indeed, Calcineurin has been shown to dephosphorylate dystrophin in vitro (Walsh et al., 1995). In addition, it has been shown that Calcineurin dephosphorylates several proteins involved in regulating exocytosis (Nishi et al., 1999).

Further, insulin secretion may be promoted by promoting the presence or activity of a serine/threonine phosphatase, which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110 or by interfering with the expression or activity of a serine/threonine kinase that phosphorylates  $\beta$ 2-syntrophin on serine residue 110. Again, that promotion or interference will lead to an interference of the binding of  $\beta$ 2-syntrophin to ICA512-TMF or ICA512-CCF and thus to the dissociation of those molecules which in turn results in the secretion of insulin. Similar to the involvement of serine residue 95, there has been no teaching or suggestion in the prior art that the phosphorylation status of serine residue 110 can be employed in the triggering of insulin secretion. Now that this specification has described the serine residues that need to be phosphorylated or dephosphorylated in order to promote insulin secretion, the enzymes (in addition to CDK5, see below) that effect the phosphorylation/dephosphorylation can be identified without further ado by screenings with specific inhibitors and activators, pharmacological modulators or RNA interference strategies.

Details about the above-mentioned screenings, especially on how phosphorylation/dephosphorylation at the specific sites are known to the skilled person. One approach is the utilisation of a genome wide RNAi library to specifically knock down different kinases and phosphatases followed by Western blot analysis. This first screening is an appropriate approach to narrow down to potential candidates. These candidate kinases could be then tested for their ability to phosphorylate  $\beta$ 2-syntrophin expressed and labeled with  $^{35}\text{S}$ -Methionine in an *in vitro* transcription and translation system. Additionally, the kinases/phosphatases identified can be overexpressed in insulinoma cells followed by analysis by western blotting of the electrophoretic profile of  $\beta$ 2-syntrophin to confirm the findings from the RNAi library. Suitable reporter assays in the screening for compounds modulating the activity of kinases or phosphatases are well known and can be purchased e.g. BioAssay Systems, CA, USA.

The pharmaceutical composition of the present invention has an enormous advantage over conventional prior art methods that rely on the promotion of insulin secretion. Namely, whereas these prior art methods rely on the promotion of insulin secretion independent of the glucose concentration in the blood and are thus potentially harmful to the patient (in particular in cases where insulin secretion is promoted in patients with lowered glucose concentrations in the blood), the

pharmaceutical composition of the present invention secures that insulin secretion is only promoted as a result of increased glucose concentrations in the blood.

Further, the present invention relates to the use of

- (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or a mimetic of the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;
- (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
- (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
- (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110

for the preparation of a pharmaceutical composition for the treatment of diabetes.

As mentioned herein above, the pharmaceutical composition to be prepared in accordance with the invention is particularly useful in the treatment of diabetes.

In a preferred embodiment of the pharmaceutical composition of the invention or the use of the invention, said mimetic is a peptidomimetic.

The chemical nature of peptidomimetics is briefly described elsewhere in this specification and is well known in the art. The use of peptidomimetics as compared to other mimetics have some particular advantages. For instance, their conformationally restrained structure allows to minimize binding to non-target receptors and enhance the activity at the desired targets. Through the addition of hydrophobic residues and/or replacement of amide bonds the transport of peptidomimetics cellular membranes can be improved. Furthermore peptidomimetics such as isosters, retro-inverso peptides, cyclic peptides and non-peptidomimetics are less susceptible to degradation by peptidases and other enzymes.

In a further preferred embodiment of the pharmaceutical composition of the invention or the use of the invention, the compound promoting the presence or activity of said protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN is an expression vector containing the coding region of said protease in expressible form.

This embodiment of the present invention may be put into practice according to conventional techniques of molecular biology including medical applications thereof such as gene therapy, and requires, optionally after cloning the gene(s) encoding the above protease and insertion of the gene or the coding sequence thereof into a vector, preferably an expression vector, the introduction into a cell. The introduction is accompanied by the expression of said protease from an expression vector. Examples of expression vectors useful in the method of the invention are described herein below.

Advantageously and as stated above, the polynucleotide/coding sequence is introduced into a vector and thus operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells or isolated fractions thereof.

Expression of said polynucleotide comprises transcription of the polynucleotide, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription

and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells (a less preferred embodiment) comprise, e.g., the *lac*, *trp* or *tac* promoter, the *lacUV5* or the *trp* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells (the more preferred embodiment) are the *AOX1* or *GAL1* promoter in yeast or the CMV- (Cytomegalovirus), SV40-, RSV-promoter (Rous sarcoma virus), the *gai10* promoter, human elongation factor 1 $\alpha$ -promoter, CMV enhancer, CaM-kinase promoter, the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedral promoter or the SV40-enhancer or a globin intron in mammalian and other animal cells. Preferred promoters are the natural promoter of the above compounds as well as promoters allowing the tissue specific expression of those compounds such as promoters active in pancreatic  $\beta$ -cells or neuronal cells. Besides elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site or the SV40, *lacZ* and AcMNPV polyhedral polyadenylation signals, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogen, as used, inter alia, in the appended examples), pSPORT1 (GIBCO BRL) or pGEMHE (Promega), or prokaryotic expression vectors, such as lambda gt11, pJOE, the pBBR1-MCS – series, pJB861, pBSMuL, pBC2, pUCPKS, pTACT1 or, preferably, the pET vector (Novagen). Preferably, said vector is an expression vector and/or a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (2001) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (2001). Alternatively, the polynucleotides or vectors can be reconstituted into liposomes for delivery to target cells.

Preferably, the vector is a plasmid, cosmid, virus, bacteriophage or another vector used e.g. conventionally in genetic engineering.

Additional non-limiting examples include prokaryotic plasmid vectors, such as the pUC-series, pBluescript (Stratagene), the pET-series of expression vectors (Novagen) or pCARTOPO (Invitrogen) and vectors compatible with an expression in mammalian cells like pREP (Invitrogen), pcDNA3 (Invitrogen), pCEP4 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2neo, pBPV-1, pDBPVMNTneo, pRSVgpt, pRSVneo, pSV2-dhfr, pIZD35, pLXIN, pSIR (Clontech), pIRES-EGFP (Clontech), pEAK-10 (Edge Biosystems) pTriEx-Hygro (Novagen) and pCINeo (Promega). Examples for plasmid vectors suitable for *Pichia pastoris* comprise e.g. the plasmids pAO815, pPIC9K and pPIC3.5K (all Invitrogen).

The polynucleotide referred to above may also be inserted into vectors such that a translational fusion with another polynucleotide is generated. The other polynucleotide may encode a protein that may e.g. increase the solubility and/or facilitate the purification of the fusion protein. Non-limiting examples include pET32, pET41, pET43. The vectors may also contain an additional expressible polynucleotide coding for one or more chaperones to facilitate correct protein folding. Suitable bacterial expression hosts comprise e. g. strains derived from BL21 (such as BL21(DE3), BL21(DE3)PlysS, BL21(DE3)RIL, BL21(DE3)PRARE) or Rosetta®.

For vector modification techniques, see Sambrook and Russel (2001), loc. cit. Generally, vectors can contain one or more origins of replication (ori) and inheritance systems for cloning or expression, one or more markers for selection in the host, e.g., antibiotic resistance, and one or more expression cassettes. Suitable origins of replication (ori) include, for example, the Col E1, the SV40 viral and the M 13 origins of replication.

The coding sequences inserted in the vector can e.g. be synthesized by standard methods, or isolated from natural sources. Ligation of the coding sequences to transcriptional regulatory elements and/or to other amino acid encoding sequences can be carried out using established methods. Transcriptional regulatory elements (parts of an expression cassette) ensuring expression in prokaryotes or eukaryotic cells are well known to those skilled in the art. These elements comprise regulatory sequences ensuring the initiation of the transcription (e. g., translation initiation codon, promoters, enhancers, and/or insulators), internal ribosomal entry sites (IRES) (Owens, Proc. Natl. Acad. Sci. USA 98 (2001), 1471-1476) and optionally

poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally associated or heterologous promoter regions. Preferably, the polynucleotide of the invention is operatively linked to such expression control sequences allowing expression in prokaryotes or eukaryotic cells. The vector may further comprise nucleotide sequences encoding secretion signals as further regulatory elements. Such sequences are well known to the person skilled in the art. Furthermore, depending on the expression system used, leader sequences capable of directing the expressed polypeptide to a cellular compartment may be added to the coding sequence of the polynucleotide of the invention. Such leader sequences are well known in the art.

Furthermore, it is preferred that the vector of the invention comprises a selectable marker. Examples of selectable markers include neomycin, ampicillin, hygromycin, kanamycin, chloramphenicol resistance and the like. Specifically-designed vectors allow the shuttling of DNA between different hosts, such as bacteria- fungal cells or bacteria-animal cells (e. g. the Gateway® system available at Invitrogen).

An expression vector according to this invention is capable of directing the replication, and the expression, of the polynucleotide and encoded enzyme. Suitable expression vectors that comprise the described regulatory elements are known in the art such as pcDNA.3 (Invitrogen, Carlsbad, CA, USA)

The nucleic acid molecules encoding the protease as described herein above may be designed for direct introduction or for introduction via liposomes, phage vectors or viral vectors (e.g. adenoviral, retroviral) into the cell. Additionally, baculoviral systems or systems based on vaccinia virus or Semliki Forest Virus can be used as eukaryotic expression system for the nucleic acid molecules of the invention.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Moreover, elements such as origin of replication, drug resistance gene, regulators (as part of an inducible promoter) may also be included. The *lac* promoter is a typical inducible promoter, useful for prokaryotic cells, which can be induced using the lactose analogue *isopropylthiol- $\beta$ -D-galactoside*. ("IPTG"). For recombinant

expression and secretion, the polynucleotide of interest may be ligated between e.g. the PelB leader signal, which directs the recombinant protein in the periplasm and the gene III in a phagemid called pHEN4 (described in Ghahroudi et al, 1997, FEBS Letters 414:521-526). Additional elements might include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from retroviruses, e.g., RSV, HTLVI, HIVI, and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells. Alternatively, the recombinant (poly)peptide can be expressed in stable cell lines that contain the gene construct integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells. The transfected nucleic acid can also be amplified to express large amounts of the encoded (poly)peptide. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al.1991, *Biochem J.* 227:277-279; Bebbington et al. 1992, *Bio/Technology* 10:169-175). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. As indicated above, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells or High Five (H5) cells; animal cells such as CHO, COS, 293 and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

In a further preferred embodiment of the pharmaceutical composition of the invention or the use of the invention; the compound promoting the presence or activity of said serine/threonine kinase is an expression vector containing the coding region of said kinase in expressible form.

In another preferred embodiment of the pharmaceutical composition of the invention or the use of the invention, the compound promoting the presence or activity of said serine/threonine phosphatase is an expression vector containing the coding region of said phosphatase in expressible form.

Regarding the employment of suitable vectors for expression of said kinase or said phosphatase, the same considerations that have been discussed herein above in connection with the expression of the protease apply.

In a different embodiment, the present invention relates to a method of enhancing insulin secretion from pancreatic  $\beta$ -cells comprising promoting in said  $\beta$ -cells the presence or activity of

- (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;
- (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
- (c) a compound promoting the presence or activity of serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) a compound interfering with the presence or activity of serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue

95;

- (e) a compound promoting the presence or activity of a phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
- (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.

In this embodiment of the invention, the same compounds that are also useful in the pharmaceutical composition of the present invention may be employed. In addition, the presence or activity of naturally occurring compounds ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN or derivatives or modifications thereof as described herein above, may be promoted. In accordance with the present invention, it was surprisingly found, as has been discussed in detail above, that these compounds can also induce insulin secretion by a different mechanism than was described in WO 2006/029728. In other terms, these compounds promote insulin secretion without the prior induction of hormone synthesis in the nucleus of the cell. This method of the present invention as well as further methods for enhancing insulin secretion from pancreatic  $\beta$ -cells can be carried out *in vivo*, *ex vivo* or *in vitro*.

In a preferred embodiment of this embodiment of the invention the promotion comprises the stable or transient expression from an exogenously introduced vector of said

- (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;
- (b) compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is

sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;

- (c) compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) compound interfering with the expression or activity of a serine/threonine phosphatases which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (e) compound promoting the presence or activity of a serine/threonine phosphatases which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
- (f) compound interfering with the expression or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.

Again, as regards the appropriate vector for expression of these proteins, sufficient guidance for the skilled person may be found in the preceding paragraphs and in the documents recited in the preceding paragraphs.

In a preferred embodiment of the pharmaceutical composition, use or method of the invention the promotion comprises reducing degradation or enhancing stability in said  $\beta$ -cells of said

- (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;
- (b) compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or

ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;

- (c) compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (e) compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or said
- (f) compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.

Reduction of the degradation comprises methods wherein proteins degrading in particular any of the proteinaceous compounds recited above are inhibited such as by RNAi or antisense oligonucleotides. In an alternative embodiment, antibodies to said degrading proteins or antibody fragments or derivatives such as Fab, F(ab')<sub>2</sub> or scFv fragments may be employed; see, for example, Harlow and Lane, "Antibodies, A laboratory manual", CSH Press 1988, Cold Spring Harbor. The goal of this embodiment of the invention is to reduce unspecific degradation, not degradation by  $\mu$ -calpain, which is, in contrast, desired in order to generate the ICA512-CCF fragment or degradation by the novel protease that specifically cleaves ICA512 and fragments thereof at the C-terminus. Preferably, the enhancement and reduction, respectively, amount to at least 25% of the amount of the compounds, preferably at least 50%, more preferred at least 75% and most preferred at least 90%. In the case of enhancement of the stability, an increase of more than 100% is advantageously envisaged. One option to enhance stability is the generation of a peptidomimetic.

In a particularly preferred embodiment of the pharmaceutical composition of the invention, the use of the invention or the method of the invention the compound interfering with the expression or activity of said phosphatase or of said kinase is, as indicated for some of the particularly preferred embodiments herein above, an siRNA, an shRNA, a ribozyme, a peptide aptamer, a nucleic acid based aptamer, a small molecule or an antibody.

For therapeutic uses, the RNA inactivation by antisense molecules or by ribozymes appears to be implementable. Both classes of compounds can be synthesized chemically or produced in conjunction with a promoter by biological expression *in vitro* or even *in vivo*.

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, are a class of 18 to 30, preferably 20 to 25, most preferred 21 to 23 or 21 nucleotide-long double-stranded RNA molecules that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway where the siRNA interferes with the expression of a specific gene. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways, e.g. as an antiviral mechanism or in shaping the chromatin structure of a genome.

SiRNAs have a well-defined structure: a short double-strand of RNA (dsRNA) with 2-nt 3' overhangs on either end. Each strand has a 5' phosphate group and a 3' hydroxyl (-OH) group. This structure is the result of processing by dicer, an enzyme that converts either long dsRNAs or small hairpin RNAs into siRNAs. SiRNAs can also be exogenously (artificially) introduced into cells to bring about the specific knockdown of a gene of interest. Essentially any gene of which the sequence is known can thus be targeted based on sequence complementarity with an appropriately tailored siRNA.

The double-stranded RNA molecule or a metabolic processing product thereof is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one RNA strand has a 5'- and/or 3'-overhang. Preferably, one end of the double-strand has a 3'-overhang from 1-5 nucleotides, more preferably from 1-3 nucleotides and most preferably 2 nucleotides. The other end may be blunt-ended or has up to 6 nucleotides 3'-overhang. In general any RNA molecule suitable to act as siRNA is envisioned in the present invention.

The most efficient silencing was so far obtained with siRNA duplexes composed of 21-nt sense and 21-nt antisense strands, paired in a manner to have a 2-nt 3'-overhang. The sequence of the 2-nt 3' overhang makes a small contribution to the specificity of target recognition restricted to the unpaired nucleotide adjacent to the first base pair (Elbashir et al. 2001). 2'-deoxynucleotides in the 3' overhangs are as

efficient as ribonucleotides, but are often cheaper to synthesize and probably more nuclease resistant.

A short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. shRNA uses a vector introduced into cells and utilizes the U6 promoter to ensure that the shRNA is always expressed. This vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs that match the siRNA that is bound to it.

RNAs of the present invention are preferably chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Suppliers of RNA synthesis reagents are Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, USA), Pierce Chemical (part of Perbio Science, Rockford, IL, USA), Glen Research (Sterling, VA, USA), ChemGenes (Ashland, MA, USA), and Cruachem (Glasgow, UK). Most conveniently, siRNAs are obtained from commercial RNA oligo synthesis suppliers, which sell RNA-synthesis products of different quality and costs. In general, the RNAs applicable in the present invention are not too difficult to synthesize and are readily provided in a quality suitable for RNAi.

A ribozyme (from ribonucleic acid enzyme, also called RNA enzyme or catalytic RNA) is an RNA molecule that catalyzes a chemical reaction. Many natural ribozymes catalyze either their own cleavage or the cleavage of other RNAs, but they have also been found to catalyze the aminotransferase activity of the ribosome.

Examples of well-characterized small self-cleaving RNAs are the hammerhead, hairpin, hepatitis delta virus, and in vitro-selected lead-dependent ribozymes. The organization of these small catalysts is contrasted to that of larger ribozymes, such as the group I intron.

The principle of catalytic self-cleavage has become well established in the last 10 years. The hammerhead ribozymes are characterized best among the RNA molecules with ribozyme activity. Since it was shown that hammerhead structures

can be integrated into heterologous RNA sequences and that ribozyme activity can thereby be transferred to these molecules, it appears that catalytic antisense sequences for almost any target sequence can be created, provided the target sequence contains a potential matching cleavage site.

The basic principle of constructing hammerhead ribozymes is as follows: An interesting region of the RNA, which contains the GUC (or CUC) triplet, is selected. Two oligonucleotide strands, each with 6 to 8 nucleotides, are taken and the catalytic hammerhead sequence is inserted between them. Molecules of this type were synthesized for numerous target sequences. They showed catalytic activity *in vitro* and in some cases also *in vivo*. The best results are usually obtained with short ribozymes and target sequences.

Aptamers are oligonucleic acid or peptide molecules that bind a specific target molecule. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist in riboswitches. Aptamers can be used for both basic research and clinical purposes as macromolecular drugs. Aptamers can be combined with ribozymes to self-cleave in the presence of their target molecule. These compound molecules have additional research, industrial and clinical applications.

More specifically, aptamers can be classified as DNA or RNA aptamers or peptide aptamers. Whereas the former consist of (usually short) strands of oligonucleotides. The latter consist of a short variable peptide domain, attached at both ends to a protein scaffold.

Nucleic acid aptamers are nucleic acid species that have been engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms.

Nucleic acid aptamers are nucleic acid species that have been engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms.

Peptide aptamers are proteins that are designed to interfere with other protein interactions inside cells. They consist of a variable peptide loop attached at both ends to a protein scaffold. This double structural constraint greatly increases the binding affinity of the peptide aptamer to levels comparable to an antibody's (nanomolar range). The variable loop length is typically comprised of 10 to 20 amino acids, and the scaffold may be any protein that has good solubility properties. Currently, the bacterial protein Thioredoxin-A is the most used scaffold protein, the variable loop being inserted within the reducing active site, which is a -Cys-Gly-Pro-Cys- loop in the wild protein, the two cysteins lateral chains being able to form a disulfide bridge. Peptide aptamer selection can be made using different systems, but the most used is currently the yeast two-hybrid system.

Aptamers offer the utility for biotechnological and therapeutic applications as they offer molecular recognition properties that rival those of the commonly used biomolecules, in particular antibodies. In addition to their discriminate recognition, aptamers offer advantages over antibodies as they can be engineered completely in a test tube, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications.

Non-modified aptamers are cleared rapidly from the bloodstream, with a half-life of minutes to hours, mainly due to nuclease degradation and clearance from the body by the kidneys, a result of the aptamer's inherently low molecular weight. Unmodified aptamer applications currently focus on treating transient conditions such as blood clotting, or treating organs such as the eye where local delivery is possible. This rapid clearance can be an advantage in applications such as *in vivo* diagnostic imaging. Several modifications, such as 2'-fluorine-substituted pyrimidines, polyethylene glycol (PEG) linkage, etc. are available to scientists with which the half-life of aptamers easily can be increased to the day or even week time scale.

A recent development is the combination of an aptamer recognizing a small compound with a hammerhead ribozyme. The conformational change induced in the aptamer upon binding the target molecule, is supposed to regulate the catalytic function of the ribozyme.

An antibody comprised in a pharmaceutical composition according to the present invention specifically binds to a compound that unfavourably influences the stability or degradation or presence of the naturally occurring compounds as described in detail herein above.

The antibody of the present invention can be, for example, polyclonal or monoclonal. The term "antibody" also comprises derivatives or fragments thereof which still retain the binding specificity. Techniques for the production of antibodies are well known in the art and described, e.g. in Harlow and Lane "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1988 and Harlow and Lane "Using Antibodies: A Laboratory Manual" Cold Spring Harbor Laboratory Press, 1999. These antibodies can be used, for example, for the immunoprecipitation, affinity purification and immunolocalization of the compounds it binds as well as for the monitoring of the presence and amount of such polypeptides, for example, in cultures of recombinant prokaryotes or eukaryotic cells or organisms. Furthermore, the antibodies are envisaged to be used in the pharmaceutical compositions of the invention.

The antibody of the invention also includes embodiments such as chimeric, single chain and humanized antibodies, as well as antibody fragments, like, inter alia, Fab fragments. Antibody fragments or derivatives further comprise  $F(ab')_2$ , Fv or scFv fragments; see, for example, Harlow and Lane (1988) and (1999), loc. cit. Various procedures are known in the art and may be used for the production of such antibodies and/or fragments. Thus, the (antibody) derivatives can be produced by peptidomimetics. Further, techniques described for the production of single chain antibodies (see, inter alia, US Patent 4,946,778) can be adapted to produce single chain antibodies specific for the compounds described above. Also, transgenic animals may be used to express humanized antibodies specific for these compounds. Most preferably, the antibody of this invention is a monoclonal antibody. For the preparation of monoclonal antibodies, any technique that provides antibodies produced by continuous cell line cultures can be used. Examples for such techniques include the hybridoma technique (Köhler and Milstein Nature 256 (1975), 495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor, Immunology Today 4 (1983), 72) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. (1985), 77-96). Surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an

epitope of the compounds described above (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). It is also envisaged in the context of this invention that the term "antibody" comprises antibody constructs that may be expressed in cells, e.g. antibody constructs which may be transfected and/or transduced via, amongst others, viruses or plasmid vectors. Once the antibody according to the present invention has been obtained by the methods of the invention, the antibody itself or the DNA encoding it can be sequenced providing for the information to recombinantly produce the antibody of the invention in small or large scale. Methods of the production of a recombinant antibody are known to the person skilled in the art.

The antibody described in the context of the invention is capable to specifically bind/interact with the compounds described above. The term "specifically binding/interacting with" as used in accordance with the present invention means that the antibody does not or essentially does not cross-react with an epitope of similar structure. Cross-reactivity of a panel of antibodies under investigation may be tested, for example, by assessing binding of said panel of antibodies under conventional conditions to the epitope of interest as well as to a number of more or less (structurally and/or functionally) closely related epitopes. Only those antibodies that bind to the epitope of interest in its relevant context (e.g. a specific motif in the structure of a protein) but do not or do not essentially bind to any of the other epitope are considered specific for the epitope of interest and thus to be antibodies in accordance with this invention. Corresponding methods are described e.g. in Harlow and Lane, 1988 and 1999, loc cit.

A small molecule according to the present invention can be organic or inorganic and has a molecular weight of up to 2000 Daltons, preferably not more than 1000 Daltons, most preferably not more than 800 Daltons. Representative small molecules are contained in the small molecule libraries. A library is a collection of stored chemicals usually used in high-throughput screenings. Each chemical is associated with information stored in a database including its chemical structure, purity, quantity, and physiochemical characteristics. Several libraries of pharmacologically active compounds (LOPAC) are commercially available (e.g. a library of 1280 compounds arrayed in sixteen 96-well-format plates is available from Sigma). Further molecules and applications are described in Wu et al. (2008).

Further, the present invention relates to a method of identifying a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells comprising contacting a compound or a plurality of compounds suspected to be useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells with a mixture of (a) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN; and (b)  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin *in vitro* under conditions that would allow binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin in the absence of the compound or the plurality of compounds and assessing binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin wherein an interference of binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin in the presence of said compound or said plurality of compounds is indicative of said compound or at least one member of said plurality of compounds being useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells.

Contacting of the test compound(s) will be done, for example, under conditions that typically or preferably resemble or are physiological conditions such as conditions achieved by incubation in physiological saline. If more than one compound is contacted with the cell and a number of compounds tests positive, it is advantageous to repeat the experiment by incubating the cell with only one compound at the time in order to identify the most promising candidate(s). In so far, a direct relation between the compound tested and its capability to enhance secretion of insulin can be established. The inclusion of an assessment step in the absence of a test compound is useful as a control.

The assessment can be established by a variety of means. For example, the binding of  $\beta$ 2-syntrophin to ICA512-TMF (or PHOGRIN) can be measured employing the experimental set-ups described in the appended examples.

Said method of identification can be accomplished in a high-throughput manner. Robotic equipment for that purpose is known in the art and available from a number of suppliers. Cells are normally grown in wells of plates containing arrays of 96, 384, 1536 or more wells. Transfer of the well-plates from incubators, addition of test compounds, optional washing steps as well as determining the read-out is performed in an automated fashion without requiring user interference using hundreds of thousands to millions of compounds in days to weeks.

Once a compound of interest has been identified, it can be further developed into a pharmaceutical agent such as by reducing its toxicity, prolonging shelf life and so on.

It is preferred in accordance with the present invention that said compound is a member of a library of compounds.

Consequently, in this and other embodiments pertaining to identification methods, the agent to be screened can be contained in libraries of small molecules, such as organic or inorganic small molecules that may be commercially available. In addition, libraries comprising antibodies or functional fragments or derivatives thereof (i.e. fragments or derivatives maintaining the binding specificity of the original antibody) may be used as a starting point in the screening process. Also, libraries of aptamers such as peptide aptamers might be employed. The skilled artisan is of course free to use any other starting point of desired compounds for use in the screen assays described throughout the specification. Suitable libraries are commercially available, for example from ChemBridge Corp., San Diego, USA.

The present invention relates in a further embodiment to a method of identifying a compound useful in the treatment of diabetes or useful as a lead compound for developing a compound useful in the treatment of diabetes comprising *in silico* assessing a crystal of ICA512 or PHOGRIN or a crystal of a fragment of ICA512 or

PHOGRIN comprising the PTP domain for a molecular structure fitting into the binding pocket of the PTP domain.

The successful identification of a protein structure is reliant on the production of high quality crystals. This requires the use of extremely pure and homogenous protein. Purity and homogeneity can be checked by methods known in the art, e.g. electrophoresis or mass spectrometry. Furthermore, in general, the more folded the protein is, i.e. the higher the ratio of folded secondary structure elements and unfolded patches, the more likely crystallization will occur. Folding of a protein can be checked e.g. with interaction studies with a known interaction partner by e.g. isothermal titration calorimetry or native gel electrophoresis methods. The proportion of secondary structure elements in the protein can be estimated using circular dichroism spectroscopy. Monodispersity, another requirement for protein crystallization, can be checked with a dynamic light scattering device. Depending on the time it will take the protein to crystallize, stability requirements may differ for each protein. The skilled person will thus understand that every protein provides its own crystallization challenge and specific protein groups, such as membrane proteins, are notoriously difficult to crystallize.

Crystal growth will occur when molecules are brought into a thermodynamically unstable state called supersaturation, achieved through the gradual removal of solvent. Nucleation, growth, and growth cessation are the three steps of crystal growth and each may require a change in experimental conditions to produce the best quality crystal.

Every protein crystallization process is hence designed and set up under a variety of conditions, adjusting experimental parameters to identify optimal crystallization conditions. These can include temperature, pH, precipitants, additives, protein concentration, and expression and purification conditions. Furthermore, the protein sample can be modified prior or during the crystallization experiment by adding modifying enzymes such as e.g. proteases which will digest the protein preferably at the respective target sites exposed to the solvent. This means that preferably parts of the protein with low organisation state and thus potentially inhibiting crystallization will be removed from the protein.

For structural determination using X-ray diffraction, not all crystals produced will diffract sufficiently. Thus, the crystallization process may have to be revisited; surface residues may have to be modified or crystallization conditions changed.

A crystallization experiment can be done in various ways. The most commonly applied methods are the hanging-drop, the sitting-drop or the micro-batch approach, all well-known to the person skilled in the art.

The surprising finding underlying this embodiment of the invention is the fact that it is the PTP domain which is involved in the interaction of, e.g. ICA512-TMF and  $\beta$ 2-syntrophin and thus leads to the enhancement of the secretion of insulin from pancreatic  $\beta$ -cells. It is of note that the PTP domain has not been correlated in the art with any possible implication in insulin secretion or the treatment of diabetes.

Further, the present invention relates to a method of identifying a compound useful for enhancing insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful enhancing insulin secretion from pancreatic  $\beta$ -cells comprising *in silico* assessing a crystal of ICA512 or PHOGRIN or a crystal of a fragment of ICA512 or PHOGRIN comprising the PTP domain for a molecular structure fitting into the binding pocket of the PTP domain.

A method of identifying a compound useful for enhancing insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful for enhancing insulin secretion from pancreatic  $\beta$ -cells comprising *in silico* assessing a crystal of  $\beta$ 2-syntrophin or a crystal of a fragment of  $\beta$ 2-syntrophin comprising the PDZ domain for a molecular structure fitting into the binding pocket of the PDZ domain constitutes another embodiment of the present invention.

In a preferred embodiment of the method of the invention, said method further comprises *in silico* or *in vitro* assessing whether (a) the compound fitting into the binding pocket of the PTP domain interferes with binding of said domain or of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN with  $\beta$ 2-syntrophin; or (b) the compound fitting into the binding pocket of the PDZ domain interferes with

binding of said domain or of  $\beta$ 2-syntrophin with ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN.

Preferred in accordance with the present invention is further a method that additionally comprises the step of testing the compound identified *in vivo*, *in vitro* or *ex vivo* for the desired capacity. Suitable tests to assess the capacity of the compound identified are described in this application and include RIA, ELISA and similar antibody based-assays, such as those employing the MSD (Meso Scale Discovery, Gaithersburg, MD, USA)

This additional step will allow significant insights in the *in vivo* applicability of the compound that tested positive in an *in vitro* assay. Parameters like toxicity, half-life etc. that have to be tested in pre-clinical trials may be assessed in this step. Suitable (non-human) animals foremost include laboratory mice and rats such as available from Charles River Laboratories etc.

Additionally, the invention envisages that the method comprises the further step of improvement or of refining the pharmacological properties of the identified agent (in the following also referred to as "compound" or "drug"), by the method as described herein above, said method comprising the optionally the steps of said methods and:

- (1) identification of the binding sites of the compound and the interacting protein by site-directed mutagenesis or chimeric protein studies;
- (2) molecular modeling of both the binding site of the compound and the binding site of the interacting protein; and
- (3) modification of the compound to improve its binding specificity for the interacting protein.

All techniques employed in the various steps of the method of the invention are conventional or can be derived by the person skilled in the art from conventional techniques without further ado. Thus, biological assays based on the herein identified nature of the interacting compound may be employed to assess the specificity or potency of the drugs wherein the increase of one or more activities of the interacting compound may be used to monitor said specificity or potency. Steps (1) and (2) can be carried out according to conventional protocols. A protocol for site directed mutagenesis (if the compound is a nucleic acid or a protein encoded

by a nucleic acid) is described in Ling MM, Robinson BH. (1997) *Anal. Biochem.* 254: 157-178. The use of homology modeling in conjunction with site-directed mutagenesis for analysis of structure-function relationships is reviewed in Szklarz and Halpert (1997) *Life Sci.* 61:2507-2520. Chimeric proteins are generated by ligation of the corresponding DNA fragments via a unique restriction site using the conventional cloning techniques described in Sambrook (1989), loc. cit.. A fusion of two DNA fragments that results in a chimeric DNA fragment encoding a chimeric protein can also be generated using the gateway-system (Life technologies), a system that is based on DNA fusion by recombination. A prominent example of molecular modeling is the structure-based design of compounds binding to HIV reverse transcriptase that is reviewed in Mao, Sudbeck, Venkatachalam and Uckun (2000). *Biochem. Pharmacol.* 60: 1251-1265.

For example, identification of the binding site of said drug by site-directed mutagenesis and chimerical protein studies can be achieved by modifications in the primary sequence of the interacting protein that affect the drug affinity; this usually allows to precisely map the binding pocket for the drug.

As regards step (2), the following protocols may be envisaged: Once the effector site for drugs has been mapped, the precise residues interacting with different parts of the drug can be identified by combination of the information obtained from mutagenesis studies (step (1)) and computer simulations of the structure of the binding site provided that the precise three-dimensional structure of the drug is known (if not, it can be predicted by computational simulation, e.g. with the programme SWISS-MODEL (<http://swissmodel.expasy.org/SWISS-MODEL.html>), or the programme MODELLER (<http://salilab.org/modeller/>) in case a three dimensional structure of a homologous protein is available). If said drug is itself a peptide, it can be also mutated to determine which residues interact with other residues in the (poly)peptide of interest.

Finally, in step (3) the drug can be modified to improve its binding affinity or its potency and specificity. If, for instance, there are electrostatic interactions between a particular residue of the interacting protein of interest and some region of the drug molecule, the overall charge in that region can be modified to increase that particular interaction.

Identification of binding sites may be assisted by computer programs. Thus, appropriate computer programs can be used for the identification of interactive

sites of a putative inhibitor and the (poly)peptide by computer assisted searches for complementary structural motifs (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the computer aided design of protein and peptides are described in the prior art, for example, in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. Modifications of the drug can be produced, for example, by peptidomimetics and other inhibitors can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive chemical modification and testing the resulting compounds. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715. Furthermore, the three-dimensional and/or crystallographic structure of activators of the expression of PTB can be used for the design of peptidomimetic activators (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

In accordance with the above, in a preferred embodiment of the method of the invention said pharmacological properties of the identified compound is further improved or refined by peptidomimetics.

The method of the invention can further include modifying a compound identified, improved or refined by the method as described herein above as a lead compound to achieve (i) modified site of action, spectrum of activity, organ specificity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of therapeutic action, duration of effect, and/or (vi) modified pharmacokinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbonic acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or

(vii) introduction of hydrophylic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substitutes to cyclic analogues, or (xiii) derivation of hydroxyl group to ketales, acetals, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetals, ketales, enolesters, oxazolidines, thiozolidines or combinations thereof; said method optionally further comprising the steps of the above described methods.

The various steps recited above are generally known in the art. They include or rely on quantitative structure-action relationship (QSAR) analyses (Kubinyi, "Hansch-Analysis and Related Approaches", VCH Verlag, Weinheim, 1992), combinatorial biochemistry, classical chemistry and others (see, for example, Holzgrabe and Bechtold, *Deutsche Apotheker Zeitung* 140(8), 813-823, 2000).

The most commonly used tool to model biological systems is molecular dynamics. Virtual screening is a computational technique to find novel drug candidates. Data from virtual screening can be used to develop predictive models in order to optimize ADMET (adsorption, distribution, metabolism, elimination, toxicology) properties of the candidate molecules. Quantitative structure-activity relationships (QSAR) represent an attempt to correlate structural or property descriptors of compounds with activities. These physicochemical descriptors, which include parameters to account for hydrophobicity, topology, electronic properties, and steric effects, are determined empirically or, more recently, by computational methods. Activities used in QSAR include chemical measurements and biological assays. Computer-assisted drug design (CADD), also called computer-assisted molecular design (CAMD), represents more recent applications of computers as tools in the drug design process. In most current applications of CADD, attempts are made to find a ligand (the putative drug) that will interact favourably with a target protein. Binding of ligand to the receptor may include hydrophobic, electrostatic, and hydrogen-bonding interactions. In addition, solvation energies of the ligand and receptor site also are important because partial to complete desolvation must occur prior to binding.

Once potential drugs have been identified by the methods described above, other molecular modelling techniques may then be applied. For example, geometry

optimization may be used to "relax" the structures and to identify low energy orientations of drugs in receptor sites. Molecular dynamics may assist in exploring the energy landscape, and free energy simulations can be used to compute the relative binding free energies of a series of putative drugs.

The invention moreover contemplates in another preferred embodiment a method wherein the compound identified and optionally further modified as indicated above is formulated into a pharmaceutical composition.

In a different preferred embodiment of the invention, the method comprises chemically synthesizing (a) the compound fitting into the binding pocket of the PTP domain and *in silico* or *in vitro* interfering with the binding of the PTP domain or of ICA512, pro-ICA512, ICA-TMF, ICA-CCF or PHOGRIN to  $\beta$ 2-syntrophin; or (b) the compound fitting into the binding pocket of the PDZ domain and *in silico* or *in vitro* interfering with the binding of the PDZ domain or  $\beta$ 2-syntrophin with ICA512, pro-ICA512, ICA-TMF, ICA-CCF or PHOGRIN.

In a further preferred embodiment of the pharmaceutical composition of the invention, the use of the invention or the method of the invention said kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95 is CDK5 (Rosales and Lee, 2006; Angelo et al, 2006). In the course of the present invention, CDK5 was shown to phosphorylate  $\beta$ 2-syntrophin on position S95. This phosphorylation results in a decreased binding affinity of  $\beta$ 2-syntrophin to ICA512, PHOGRIN and functional fragments thereof.

In a further preferred embodiment of the pharmaceutical composition, use or method of the invention for enhancing insulin secretion, said compound is a small molecule, a peptide, an antibody, a peptide aptamer, a nucleic acid aptamer, a glucose metabolite, an anticalin, an siRNA, an shRNA, a ribozyme, an antisense nucleic acid molecule or a mimetic, preferably a peptidomimetic.

SiRNA, shRNA, antisense nucleic acid molecules and ribozymes only come into play if the molecule to be interfered with is a nucleic acid molecule such as an mRNA molecule. Antisense nucleic acid molecules may be DNA or RNA molecules.

The term "antibody", as used in accordance with the present invention includes antibody fragments or derivatives, such as Fab' fragments, F(ab<sub>2</sub>)', scFvs, humanized or chimeric antibodies and has been described in more detail above.

Peptide aptamers are small molecule drugs that inhibit intracellular protein interactions (Hoppe-Seyler F, Crnkovic-Mertens I, Tomai E, Butz K. 2004. Peptide aptamers: specific inhibitors of protein function. *Curr Mol Med*. 2004 Aug;4(5):529-38) and have been further described above. Anticalins are engineered proteins with antibody-like ligand-binding functions derived from natural lipocalins as a scaffold (Skerra A. 2001 'Anticalins': a new class of engineered ligand-binding proteins with antibody-like properties. *J Biotechnol*. 74:257-75).

Further, the present invention relates to a glucose metabolite or a structurally related compound/derivative thereof for the preparation of a pharmaceutical composition for the treatment of diabetes.

As has been described herein above, one surprising finding in the present invention is that it is the PTP domain that is involved in the interaction of, e.g. ICA512-TMF and  $\beta$ 2-syntrophin and thus leads to the enhancement of the secretion of insulin from pancreatic  $\beta$ -cells.

The requirement of the glucose metabolite or structurally related compound/derivative is thus to fit into the catalytically inactive pocket of the PTP of ICA512, PHOGRIN or functional fragments thereof to modulate their activity such that their interaction with  $\beta$ 2-syntrophin is reduced or abolished.

The term "derivative" as used in accordance with the present invention relates to a compound derived from glucose metabolites in a way that they fit into the catalytically inactive pocket of the PTB of ICA512, PHOGRIN or functional analogues thereof. Thus, any chemical modification to a glucose metabolite, in particular to glyceraldehyde-3-phosphate, dihydroxyacetonephosphate or 1,3-diphosphoglycerate that conserves or enhances the binding properties/binding strength of the respective molecule to said catalytically inactive pocket falls within the scope of the present invention. Methods to test whether a compound falls under the scope of the present

invention are well known to the skilled person, disclosed in the present invention and described in detail above and in the appended examples.

Such methods include e.g. co-immunoprecipitation, native gel electrophoresis and isothermal titration calorimetry to assess the interaction of ICA512, PHOGRIN or functional fragments thereof to  $\beta$ 2-syntrophin in the presence and absence of a candidate glucose metabolite or derivative thereof.

Co-immunoprecipitation is a technique for the analysis of protein-protein interactions. The procedure includes binding of the protein of interest to an antibody specific to said protein under physiological conditions. The antibody-protein complex is then precipitated usually using protein-G or protein-A sepharose that binds most antibodies. If there are any protein/molecules that bind to the protein of interest with a sufficient strength, they will also be precipitated. Co-precipitated proteins can be identified by Western blot analysis or by sequencing a purified protein band.

Native gel electrophoresis is used to determine whether two proteins interact. A complex of two interacting proteins shows a different electrophoretic mobility as compared to the single proteins, apart from the fact that in the latter case, two bands would usually be observed on the gel instead of only one in the presence of the complex. A weak interaction of the proteins may also result in three bands corresponding to each of the single proteins and the complex so that the application of a different method may be necessary to confirm and/or quantify the interaction.

Isothermal titration calorimetry is used to study interactions between molecules (reviewed in Pierce et al., 1999). These can be protein-ligand (e.g. peptides or DNA) interactions as well as protein-protein interactions. Not only can the overall binding of two molecules easily be detected, but the stoichiometry  $n$ , the binding affinity ( $K_a$ ) and the enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) changes of a given reaction can also be determined in a straightforward manner. The total concentration of all but one of the reactants remains constant throughout the experiment. Non-linear least square fitting of the calorimetric data from a single experiment, where heat is the only dependent variable, allows an estimation of the thermodynamic parameters.

ITC is used to measure the heat evolved or absorbed when e.g. a protein and a ligand are mixed. In general, the ligand, which has about ten-fold higher concentration than the

protein is titrated in small volumes into a large volume of protein solution. The method is non-destructive so that the protein and the ligand can be recovered after the measurement. Using this method, not only the binding affinity of glucose metabolites and derivatives to the PTP domain can be evaluated in a straightforward manner but also that of the complex of the glucose metabolite or derivative with the PTP domain to  $\beta$ 2-syntrophin.

In a preferred embodiment of the use of the invention said glucose metabolite is glyceraldehyde-3-phosphate.

In a most preferred embodiment of the invention, said diabetes is type-2 diabetes.

The figures show:

**Figure 1. Calpain-1-mediated cleavage of ICA512 regulates insulin secretion.**

**(A)** Insulin secretion stimulation index (SI) of pancreatic islets from *ICA512<sup>+/+</sup>* or *ICA512<sup>-/-</sup>* mice treated with (+) or without (-) 60 $\mu$ M Calpeptin. The SI of *ICA512<sup>+/+</sup>* islets was equaled to 100%. **(B)** SI of INS-1E cells non-transfected, or transfected stably or transiently with 2 or 5 $\mu$ g *ICA512-GFP*, and then treated or untreated with 60 $\mu$ M Calpeptin. The SI of non-transfected INS-1E cells was equaled to 100%. **(C)** SI of INS-1E cells stimulated with high glucose (25 mM, HG), high potassium (55 mM, HK), or both (HGHK). Cells were treated with or without 60 $\mu$ M Calpeptin, 100 $\mu$ M Cycloheximide (CHX), or both. The SI of cells stimulated with HGHK was equaled to 100%. **(D)** Ratio of secreted vs. total insulin of *ICA512<sup>+/+</sup>* (black columns) or *ICA512<sup>-/-</sup>* (gray columns) mouse islets kept at rest or stimulated with HGHK or HK and treated with or without 60 $\mu$ M Calpeptin. The ratio in resting, untreated *ICA512<sup>+/+</sup>* islets, was equaled to 1. Error bars show mean  $\pm$  SD. All the results are from at  $\geq 3$  independent experiments except (D) which is from 2 independent experiments performed in triplicate. Single asterisk (\*):  $p < 0.05$ , double asterisks (\*\*):  $p < 0.01$ , triple asterisks (\*\*\*):  $p < 0.005$ .

**Figure 2. ICA512-CCF stimulates insulin secretion.** (A) Ratio of secreted vs. total insulin from INS-1E cells non-transfected, or transiently transfected with 5 $\mu$ g *GFP* or 2 or 5  $\mu$ g *ICA512-CCF-GFP* plasmids. Cells were kept at rest or stimulated with HGHK and treated with or without 60 $\mu$ M Calpeptin. The ratio in electroporated, resting INS-1E cells was equaled to 1. (B) Western blottings with anti-GFP (top) or anti-ICA512 (bottom) antibodies on total protein extracts from INS-r3 cells transiently transfected with tetracycline inducible *ICA512-CCF-GFP*. Cells were treated with or without 500ng/ml Doxycyclin for various time intervals as indicated. (C and D) Insulin secretion (C) and content (D) of INS-r3 cells treated as in (B). Results in (C) and (D) are from 3 independent experiments, each in triplicate. (E-H) Confocal Z-sections (0.5  $\mu$ m) of INS-1E cells immunolabeled with anti-insulin antibody (pseudored). Nuclei were counterstained with DAPI (pseudoblue). Arrows and arrowheads point to the accumulation of insulin immunoreactivity in proximity of the plasma membrane and the perinuclear region, respectively. In (F-H) cells were transiently transfected with 5 $\mu$ g *ICA512-CCF-GFP* (F, H) or *GFP* (G) (pseudogreen). In (H) cells were co-transfected with *tetanus toxin light chain* as described (Trajkovski et al., 2004). Bars in (E-H): 10  $\mu$ m. Error bars in (A), (C), and (D) show mean  $\pm$  SD. The results are from  $\geq 3$  independent experiments performed in triplicate. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.005$ .

**Figure 3. ICA512-CCF dimerizes with ICA512-TMF.** (A) Western blottings with mouse anti-GFP (top) or anti-ICA512 (bottom) antibodies on total protein extracts (lanes 1 and 2), or on immunoprecipitates (IP) with rabbit anti-ICA512ecto (lanes 3 and 4) or control (lanes 5 and 6) immunoglobulins from INS-1E transfected or not with 5 $\mu$ g *ICA512-CCF-GFP*. (B) Western blottings with mouse anti-ICA512-HM1 (Mziaut et al., 2006) (top) or anti-GFP (bottom) antibodies on cytosolic protein extracts (lanes 1 to 4) or immunoprecipitates with goat control (lanes 5 to 8) or anti-GFP (lanes 9 to 12) immunoglobulins from resting or HGHK-stimulated INS-1E cells. Cells were either nontransfected or transfected with 5 $\mu$ g *ICA512-GFP*. (C) SDS-PAGE and autoradiography of in-vitro transcribed and translated  $^{35}$ S-ICA512700-979 (top) or  $^{35}$ S-luciferase (bottom) pulled down with glutathione sepharose beads coupled to 1 $\mu$ g of the following recombinant proteins: GST-ICA512-601-979, GST-ICA512-663-979, GST-ICA512-700-979, GSTICA512-700-959, GST-ICA512 extracellular, GST-ICA512-800-979 or GST. The input of  $^{35}$ S-ICA512-700-979 and

<sup>35</sup>S-luciferase for the pull-down assays is shown in lanes 1. **(D)** Western blotting with anti-His antibodies of bacterially expressed ICA512-601-979-His pulled down with glutathione sepharose beads coupled to 1 µg of GST-ICA512-601-979, GST-ICA512-663-979 or GST.

**Figure 4. ICA512-CCF dissociates ICA512-TMF from β2-syntrophin.** **(A)** Western blottings with mouse anti-ICA512 (top panel) or anti-GFP (middle and bottom panels) antibodies on total protein extracts from resting or HGHK-stimulated INS-1 cells stably transfected with *GFP* or *GFP-β2-syntrophin*. **(B)** Western blottings with mouse anti-ICA512 (first and fourth panels from the top), anti-GFP (second and last panels from the top), or anti-γ-tubulin (third panel from the top) antibodies on total protein extracts (first three panels from the top) or immunoprecipitates with anti-GFP antibodies from resting or HGHK-stimulated INS-1E cells. Cells were stably transfected with *GFP* or *GFP-β2-syntrophin* and treated with or without 60µM Calpeptin. **(C)** SDS-PAGE and autoradiography of in-vitro transcribed and translated <sup>35</sup>S-β2-syntrophin (top panel), and western blottings with anti-His antibodies of bacterially expressed ICA512601-979-His (bottom panel), pulled down with glutathione sepharose beads coupled to 1 µg GSTICA512601-979. **(D)** Western blottings with anti-GFP (top panels), anti-ICA512 (second panels from the top), and anti-HA (third panels from the top) or γ-tubulin (bottom left panel) antibodies on total protein extracts (input, left panels) or immunoprecipitates (IP) with goat control (lanes 6-10) or anti-GFP (lanes 11-15) immunoglobulins (right panels) from INS-1E cells stably co-transfected stably with *GFP* or *GFP-β2-syntrophin* and transiently with 5µg *HA3-ICA512-CCF*, *ICA512-CCF-HA3* or control *HA3* vectors. **(E)** Western blotting with the anti-HA antibody on total protein extracts (lanes 1 and 2); or immunoprecipitates with goat control (lanes 3 and 4) and anti-GFP (lanes 5 and 6) immunoglobulins from INS-1E cells transfected stably with *GFP-β2-syntrophin* or *ICA512-GFP* and transiently with 5µg *HA3-ICA512-CCF*. **(F)** Ratio of secreted vs. total insulin from INS-1E cells transiently transfected with 5 µg *HA3-ICA512-CCF*, *ICA512-CCF-HA3*, or the control *pNH3* and *MoHA3* vectors used for the generation of the HA-tagged *ICA512-CCF* constructs. The ratio of cells transfected with *pNH3* and *MoHA3* was equaled to 1. **(G)** SI of insulin secretion from INS-1E cells transiently transfected with vectors for the individual or combined knockdown of *ICA512* (*pGENE-ICA512*), *β2-syntrophin* (*pGENE-β2-syntrophin*), or the control

*pGENE-Clip*. Cells were cotransfected with 5 $\mu$ g *GFP- $\beta$ 2-syntrophin* or *ICA512-GFP*, as indicated. The SI in cells transfected with *pGENE-Clip* was equaled to 100%. The results are from  $\geq 3$  independent experiments performed in triplicate. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.005$ .

**Figure 5. ICA512-CCF increases granule motility.** (a-f) Frames from TIRFM movies tracking the motion of CgB-mRFP1<sup>+</sup> granules in resting INS-1 cells expressing GFP (A, C, E) or ICA512-CCF-GFP (B, D, F). (A and B) show the raw images, while (C and D) show the tracked granules, as recognized by the Motiontracking II software. In (E and F) the motion of individual CgB-mRFP1<sup>+</sup> granules tracked for 50 sec. is displayed with a blue line. (g) Density of granules in resting INS-1 cells expressed as number of granules per 100  $\mu$ m<sup>2</sup>. (h and i) Mean speed velocity (h) and square displacement (i) of CgBmRFP1<sup>+</sup> granules in resting INS-1 cells expressing GFP (black traces) or ICA512-CCFGFP (red traces). In (h) the histogram bins were sampled uniformly in the logarithmic scale to better present the distribution difference at low speed. The sum of all bins equals 1 for both histograms. In (i) the green and blue lines show the weighted linear fit of the 3 initial mean square displacement points. Bars: 5  $\mu$ m.

**Figure 6. Regulation of granule turnover by ICA512.** A schematic representation of a model illustrating how stimuli inducing the release of insulin (such as glucose, 1) regulate granule turnover through the calpain-1-mediated cleavage of ICA512-TMF at the plasma membrane. The resulting cleaved cytoplasmic fragment, termed ICA512-CCF (2), disrupts the ICA512-TMF/ $\beta$ 2-syntrophin complex by binding to either component of this complex (3 and 3'). The consequential displacement of granules from cortical actin increases their mobility and likelihood to approach sites competent for exocytosis (4). Progressive depletion of granules, increases the probability of ICA512-CCF to reach the nucleus (5), thereby increasing the transcription of granule genes by enhancing the activity of STAT-5 (6), as previously shown (Trajkovski et al., 2004), and thus granule biogenesis (7). For graphic reasons granules tethered to cortical actin filaments via the ICA512-TMF/ $\beta$ 2-syntrophin complex appear distant from the plasma membrane, although in reality they are located in very close proximity to it.

**Figure 7.** (A) Schematic domain representation of human ICA512. (B) Western blotting with the anti-ICA512-HM1 antibody on total protein extracts from resting or stimulated INS-1E cells as in Fig. 1d, treated with or without 60 $\mu$ M Calpeptin. The migration of standard molecular weight (kDa) proteins is indicated on the right.

**Figure 8.** (A) Confocal Z-sections (0.5  $\mu$ m) of INS-1E cells transiently transfected with 5 $\mu$ g *ICA512-CCF-GFP* (pseudogreen) double immunostained with antiinsulin (pseudored) and anti-TGN38 (pseudoblue) antibodies. (B) Ratio of secreted vs. total insulin of INS-1E cells transfected with *pNH3*, *HA3-ICA512-CCF* or *ICA512-CCFGFP*. The ratio in cells transfected with *pNH3* was equaled to 1. (C) Confocal Z-sections (0.5  $\mu$ m) of INS-1E cells transiently transfected with 5 $\mu$ g *HA3-ICA512-CCF* immunolabeled with an anti-insulin antibody (pseudored). The nuclei are counterstained with DAPI (pseudoblue). In (A) and (C) the granules near the plasma membrane and the Golgi complex region are indicated with arrows and arrowheads, respectively. Bars: 10  $\mu$ m.

**Figure 9.** (A) Western blottings with anti-ICA512-HM1, anti-CPE and anti-Glut2 antibodies on INS-1E cell fractions separated on a continuous 0,4-2M sucrose density gradient. The arrows point to the band with the expected mobility of ICA512-CCF. (B) Western blotting with anti-ICA512-HM1 on immunoprecipitates (IP) obtained with goat control (lanes 1-3), or anti-GFP immunoglobulins from total protein extracts of resting or HGHK-stimulated INS-1E cells transfected with or without 5 $\mu$ g *ICA512-GFP*, and treated with or without 60 $\mu$ M Calpeptin. The arrow points to co-immunoprecipitated endogenous ICA512-CCF. (C) Coomassie Blue staining of bacterially expressed GST-ICA512-601-979, GST-ICA512-663-979, GST-ICA512-700-979, GST-ICA512-700-959. (D) SDS-PAGE and autoradiography of in-vitro transcribed and translated  $^{35}$ S-ICA512-601-979, ICA512-700-979, and  $^{35}$ S-Luciferase used as starting material in figure 3c. (E) SDS-PAGE and autoradiography of in-vitro transcribed and translated  $^{35}$ S-ICA512-601-979 pulled down with glutathione sepharose beads coupled to 1  $\mu$ g GST-ICA512-601-979, GST-ICA512-663-979, GST-ICA512-700-979, GST-ICA512-700-959, GST-ICA512extracellular, GST-ICA512-800-979, or GST alone. The starting material for the pull-down is shown in lane 1.

**Figure 10.** (a) left: Western blottings with anti-ICA512 (top panel), anti- $\beta$ 2-syntrophin (middle panel), or anti- $\beta$ -tubulin (bottom panel) antibodies on total protein extracts from INS-1E cells transiently transfected with pGENE, pGENE-ICA512, or pGENE- $\beta$ 2-syntrophin as in figure 4g. Right: Western blottings with anti- $\beta$ 2-syntrophin (top panel), anti-ICA512 (middle panel), or anti-GFP (bottom panel) antibodies on total protein extracts (lanes 1 and 2), and immunoprecipitates obtained with anti-GFP (lanes 3 and 4) antibodies from INS-1 cells stably transfected with ICA512-CCF-GFP. (b) SI of insulin secretion from INS-1E cells transiently transfected with vectors for the knockdown of rat *ICA512* (left), rat  *$\beta$ 2-syntrophin* (right), and the control empty vector for RNAi (left and right). The specificity of the knockdown was confirmed by over-expression of RNAi degradation resistant forms, i.e. human *ICA512-GFP* (left) or mouse *GFP- $\beta$ 2-syntrophin* (right). The SI in cells transfected with the control vector equals 100%. The results are from at least 3 independent experiments performed in triplicate. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.005$ .

**Figure 11.** (a) Staining of INS-1 cells expressing GFP or ICA512-CCF-GFP (pseudogreen) with phalloidin-rhodamine (pseudored). Nuclei were counterstained with DAPI (pseudoblue). Bars: 10 $\mu$ m. (b) Top row: Co-localization of CgB-mRFP1 (pseudored) and insulin (pseudogreen) in stable CgB-mRFP1 INS-1 cells. The area in the inset is magnified in the bottom row. Arrows point to granules positive for insulin and CgB-mRFP1. Nuclei were counterstained with DAPI (blue). Bars: 5 $\mu$ m.

The examples illustrate the invention

### Example 1: ICA512 cleavage regulates exocytosis

The calpain inhibitor calpeptin reduces the cleavage of ICA512-TMF in stimulated INS-1 cells (Ort et al., 2001; Trajkovski et al., 2004) and also in the more glucose responsive INS-1E cells (Merglen et al., 2004) (Fig. 7B). Calpeptin also inhibited insulin secretion by  $\geq 70\%$  from INS-1 cells (Ort et al., 2001), INS-1E cells (Fig. 1B, bars 1 and 2) and from purified mouse pancreatic islets (Fig. 1A, bars 1 and 2)

following stimulation with high glucose (25 mM, HG) for 120 or 90 minutes, respectively. Consistent with previous studies (Saeki et al., 2002), the insulin stimulation index of *ICA512*<sup>-/-</sup> islets was reduced by 58% ± 8.7 compared to *ICA512*<sup>+/+</sup> islets (Fig. 1A, bars 1 and 3). Strikingly, calpeptin did not further decrease insulin secretion from *ICA512*<sup>-/-</sup> islets (Fig. 1A, bar 4), pointing to the calpain-1-mediated cleavage of ICA512-TMF being involved in regulation of insulin secretion. Accordingly, both stable and transient overexpression of ICA512-GFP in INS-1E cells enhanced their insulin release in a dose-dependent and calpeptin-sensitive fashion (Fig. 1B). Since calpeptin did not inhibit the increase of pro-ICA512 which is observed within 2 hours following stimulation (Mziaut et al., 2006) (Fig. 7B), and which results from the activation of post-transcriptional mechanisms (Knoch et al., 2004), it seems likely that cleavage of ICA512-TMF promotes insulin secretion independently from its role on gene transcription and granule biogenesis. Indeed, block of protein synthesis with cycloheximide (Fig. 1C, bars 7-10), unlike calpeptin (Fig. 1C, bars 4-6 and 10), did not significantly reduce insulin secretion following stimulation with HG, high potassium (55 mM, HK) or both. Finally, depolarization of  $\beta$ -cells with HK alone, which triggers granule exocytosis and cleavage of ICA512-TMF by inducing  $\text{Ca}^{2+}$ -entry (Ort et al., 2001; Trajkovski et al., 2004) (Fig. 7B), but not the biosynthesis of preproinsulin (Steiner et al., 2001) and pro-ICA512 (Ort et al., 2001; Trajkovski et al., 2004) (Fig. 7B), increased ~5 fold insulin secretion from *ICA512*<sup>+/+</sup> islets (Fig. 1D, bars 1 and 5), but not from *ICA512*<sup>-/-</sup> islets (Fig. 1D, bars 2 and 6). Moreover, this increment was calpeptin-sensitive in *ICA512*<sup>+/+</sup> islets (Fig. 1D, bars 5 and 7), but not in *ICA512*<sup>-/-</sup> islets (Fig. 1D, bars 6 and 8). Taken together, these results demonstrate that cleavage of ICA512-TMF is a key event for regulating the  $\text{Ca}^{2+}$ -dependent exocytosis of insulin granules.

### **Example 2: ICA512-CCF directly affects secretion**

Next, we evaluated the direct effect of ICA512-CCF on insulin secretion. Overexpression of ICA512-CCF fused to green fluorescent protein (ICA512-CCF-GFP) (Fig. 2A, bars 2, 7, and 9), but not GFP (Fig. 2A, bars 2 and 5) increased stimulated insulin release in a concentration dependent fashion. Strikingly, calpeptin inhibited stimulated insulin secretion by 77% ± 5.3 and 75% ± 13.0 from control (Fig. 2A, bar 4) and GFP-INS-1E cells (Fig. 2A, bars 5 and 6), respectively, while it had

only a modest (Fig. 2A, bars 7 and 8), or no effect (Fig. 2A, bar 9 and 10) in *ICA512-CCF-GFP-INS-1E* cells, hence indicating that ICA512-CCF enhances granule exocytosis directly, and not through the regulation of proteins whose activity on secretion is calyptin-sensitive. To further investigate its relationship with insulin secretion, ICA512-CCF was expressed in the INSr3 cells (Wang and Iynedjian, 1997) in a tetracycline-inducible fashion. ICA512-CCF GFP was first detectable 16 hours after its induction with doxycyclin, and maximally expressed after 24 hours (Fig. 2B). Its appearance correlated at first with decreased levels of endogenous ICA512-TMF (Fig. 2B, lane 2) followed by increased pro-ICA512 and ICA512-TMF levels (Fig. 2B, lane 3). In parallel, insulin secretion increased by  $65\% \pm 6.54$  (Fig. 2C, point 1), before the increment in insulin content (Fig. 2D, point 1 and 2), indicating that the direct effect of ICA512-CCF on secretion precedes its role on granule biogenesis.

ICA512-CCF-GFP dramatically altered the intracellular distribution of insulin secretory granules (Fig. 2E and F). In INS-1E cells expressing ICA512-CCF-GFP, but not GFP alone, insulin immunoreactivity was reduced at the cell periphery (Fig. 2F), where granules typically accumulate (Fig. 2E and G, arrows), while it was increased throughout the cells and especially in the Golgi region (Fig. 2F, arrowheads, and Fig. 8A). The depletion of peripheral granules in *ICA512-CCF-GFP-INS-1E* cells was prevented by the co-transfection of tetanus toxin light chain, which blocks granule exocytosis (Fig. 2H, arrows), but not with an inactive tetanus toxin light chain mutant (not shown). Similar effects were observed when expressing ICA512-CCF N-terminally tagged with a triple HA epitope (HA3-ICA512-CCF). HA3-ICA512-CCF was also enriched in the nucleus (Fig. 8C), and stimulated insulin secretion slightly more than ICA512-CCF-GFP (Fig. 8B). Moreover, its expression increased insulin immunoreactivity in the Golgi region (Fig. 8C, arrowheads), while decreasing it at the periphery (Fig. 8C, arrows). Depletion of cortical granules and accumulation of insulin along the secretory pathway are consistent with ICA512-CCF having a dual role in enhancing granule exocytosis while promoting their biogenesis.

### **Example 3: ICA512-CCF and ICA512-TMF homodimerize**

We then attempted to identify how ICA512-CCF induces granule exocytosis. Separation of INS-1E cell extracts by sucrose density gradients revealed that fractions enriched in the granule markers ICA512-TMF and carboxypeptidase H

contained a minor protein reactive with an antibody against ICA512-CCF (Mziaut et al., 2006) and the expected size for ICA512-CCF of ~36 kDa (Fig. 9A, arrow). Since the ICA512 cytoplasmic domain can form homodimers *in vitro* and in transfected fibroblasts (Gross et al., 2002), ICA512-CCF could potentially bind through ICA512-TMF to granule membranes. Supporting this hypothesis, ICA512-CCF-GFP was selectively coimmunoprecipitated using an antibody against the ICA512-TMF ectodomain (Solimena et al., 1996), but not with control IgG (Fig. 3A). Likewise, endogenous ICA512-CCF was immunoprecipitated with the anti-GFP antibody from stimulated, but not from resting (Fig. 3B) or calpeptin-treated (Fig. 9B, arrow) INS-1E cells expressing ICA512-GFP. On the other hand, we neither detected pro-ICA512 nor ICA512-TMF homodimers.

To map the dimerization domain, the cytoplasmic (ICA512601-979) and PTP (ICA512700-979) portions of ICA512 and firefly luciferase as a control protein were *in vitro* transcribed and translated in the presence of 35S-methionine and used for pull-down assays (Fig. 3C, and Fig. 9D and 9E) with various recombinant GST-ICA512 fusion proteins (Fig. 9C). Both 35S-ICA512601-979 and 35S-ICA512700-979 bound similarly to GST-ICA512601-979, GST-ICA512663-979, GST-ICA512700-979 and GST-ICA512700-959, but not to GST-ICA512800-979, which lacks part of the PTP domain, or GST-ICA512extracellular, while the control protein 35S-Luciferase, did not heterodimerize with GST-ICA512601-979 (Fig. 3C, lower panel). These data indicate that the PTP domain without its flanking N- and C-terminal PDZ domain binding regions is necessary and sufficient for ICA512 homodimerization (Fig. 3C and Fig. 9E). Moreover, bacterially expressed ICA512601-979 tagged with a 6-histidine epitope (ICA512601-979-His) did also bind to GST-ICA512601-979 and GST-ICA512663-979 (Fig. 3D), thus demonstrating that ICA512 dimerization does not depend on other proteins.

#### **Example 4: ICA512-CCF displaces ICA512-TMF from $\beta$ 2-syntrophin**

Considering that ICA512-TMF binds  $\beta$ 2-syntrophin (Ort et al., 2000; Ort et al., 2001) and that ICA512-CCF dimerizes with ICA512-TMF, as shown here, we asked whether ICA512-CCF affects the ICA512-TMF/ $\beta$ 2-syntrophin complex by binding either protein. We first demonstrated that consistent with  $\beta$ 2-syntrophin interacting

with ICA512-TMF and protecting it from cleavage (Ort et al., 2001), over-expression of  $\beta$ 2-syntrophin-GFP increased ICA512-TMF levels in both resting and stimulated INS-1E cells (Fig. 4A, lanes 3 and 4). Conversely, knockdown of  $\beta$ 2-syntrophin by RNAi reduced ICA512-TMF expression and vice versa (Fig. 10a, left panel). Extending previous findings (Ort et al., 2001), we then found that ICA512-TMF co-immunoprecipitated with GFP- $\beta$ 2-syntrophin from extracts of INS-1E cells kept in resting buffer or stimulated in Trajkovski et al, 2007 12 the presence of calpeptin (Fig. 4B, lanes 4 and 6), but not from stimulated cells (Fig. 4B, lane 5). These data support the possibility that ICA512-CCF disrupt the interaction of ICA512-TMF with  $\beta$ 2-syntrophin.

We subsequently investigated whether ICA512-CCF affects the interaction of ICA512-TMF with  $\beta$ 2-syntrophin (Ort et al., 2000; Ort et al., 2001) by binding to either protein. Additional evidence of the close relationship between  $\beta$ 2-syntrophin and ICA512-TMF was that overexpression of the former fused to GFP increased the levels of the latter, both in resting and stimulated cells (Fig. 4A, lanes 3 and 4). Conversely, knockdown of  $\beta$ 2-syntrophin by RNAi reduced the expression of ICA512-TMF and vice versa (Fig. 10a). Also consistent with preliminary findings (Ort et al., 2001) ICA512-TMF was co-immunoprecipitated with GFP- $\beta$ 2-syntrophin from extracts of resting (Fig. 4B, lane 4), but not stimulated (Fig. 4B, lane 5), INS-1E cells. However, ICA512-TMF was still co-immunoprecipitated with GFP- $\beta$ 2-syntrophin upon calpeptin treatment of stimulated cells (Fig. 4B, lane 6), hence suggesting that ICA512-CCF modulates the dissociation of ICA512-TMF from  $\beta$ 2-syntrophin. To test this hypothesis, we analyzed how homodimerization of ICA512 affects its binding to  $^{35}\text{S}$ - $\beta$ 2-syntrophin by pull-down assays. These analyses clearly showed that increased binding of ICA512-601-979-His to GST-ICA512-601-979 (Fig. 3C, bottom) proportionally decreased the interaction of GST-ICA512-601-979 with  $^{35}\text{S}$ - $\beta$ 2-syntrophin (Fig. 3C, top). To further test this scenario within INS-1E cells, ICA512-CCF either N-terminally (HA3-ICA512-CCF) or C-terminally (ICA512-CCF-HA3) tagged with a triple HA epitope was co-expressed with GFP- $\beta$ 2-syntrophin or GFP (Fig. 4D, input). Remarkably, by immunoblotting with the anti-HA antibody HA3-ICA512-CCF appeared as a close doublet (Fig. 4D, lower middle panel, lanes 2 and 4), suggesting that within cells its C-terminus which includes the binding site for the PDZ of  $\beta$ 2-syntrophin (Fig. 8A) is proteolytically removed (Ort et al., 2000). This

cleavage could be precluded by fusion of the C-terminus with a tag, as in ICA512-CCF-HA3 and ICA512-CCF-GFP, which always appeared as single bands by immunoblotting (Fig. 4D, lower middle panel, lane 5 and Fig. 3A, top panel, lanes 2 and 4). As we expected, the association of ICA512-TMF with GFP- $\beta$ 2-syntrophin was strongly reduced upon expression of HA3-ICA512-CCF (Fig. 4D, middle panel, lanes 13 and 14), and even more so by ICA512-CCF-HA3, whose C-terminal tag conceivably hampers its interaction with GFP- $\beta$ 2-syntrophin (Fig. 4D, middle panel, lane 15). Co-immunoprecipitations with an antibody directed against the ectodomain of ICA512-TMF further indicated that the interaction of endogenous  $\beta$ 2-syntrophin with ICA512-TMF is also reduced upon expression of ICA512-CCF-GFP relatively to GFP alone (Fig. 10a, left column, top panel, lanes 3 and 4).

Unlike ICA512-TMF (Fig. 4E, lane 5), GFP- $\beta$ 2-syntrophin interacted preferentially with the higher as compared to the lower of the two HA3-ICA512-CCF species (Fig. 4D, bottom panel, lane 14 and Fig. 4E, lane 6). This result can be explained by considering that the lower HA3-ICA512-CCF species, despite the likely removal of the C-terminal binding site for  $\beta$ 2-syntrophin, still contains a PDZ binding site N-terminally to the PTP domain (Ort et al., 2000) (Fig. 7A). By favoring the interaction with ICA512-TMF over  $\beta$ 2-syntrophin, the C-terminal cleavage of ICA512-CCF could represent a novel mechanism for promoting the mobilization of granules. Accordingly, ICA512-CCF-HA3, which did not bind  $\beta$ 2-syntrophin and induced a stronger dissociation of the ICA512-TMF/ $\beta$ 2-syntrophin complex (Fig. 4D, lane 15), enhanced insulin secretion more than HA3-ICA512-CCF (Fig. 4F). Corroborating the role of the ICA512-TMF/ $\beta$ 2-syntrophin complex in regulating the retention of granules and their exocytosis, knockdown of  $\beta$ 2-syntrophin reduced the insulin stimulation index to  $34\% \pm 1.0$  compared to control cells (Fig. 4G, bar 3). As expected (Harashima et al., 2005; Saeki et al., 2002), knockdown of ICA512 also decreased stimulated insulin secretion (Fig. 4G, bar 2), which was further diminished upon knockdown of both proteins (Fig. 4G, bar 4). These effects were specific, as they could be largely rescued by the overexpression of human ICA512- GFP and mouse GFP- $\beta$ 2-syntrophin (Fig. 10b), which were resistant to RNAi due to several mismatches between their nucleotide sequences and that of their rat counterparts. Conversely, overexpression of GFP- $\beta$ 2-syntrophin increased the insulin stimulation index by  $258\% \pm 80$  compared to control cells (Fig. 4G, bar 5) and most importantly, this increment was abolished upon

knockdown of ICA512 (Fig. 4G, bar 6). Likewise, knockdown of  $\beta$ 2-syntrophin reduced stimulated insulin secretion from ICA512-GFP over-expressing cells (Fig. 4G, bars 7 and 8), altogether demonstrating that the two proteins act on the same pathway.

#### **Example 5: ICA512-CCF increases granule mobility**

ICA512-CCF could increase granule exocytosis by affecting the integrity of the cortical actin cytoskeleton, which may act as a barrier that prevents granules from having access Trajkovski et al, 2007 15 to the plasma membrane. However, staining of F-actin with phalloidin revealed no detectable alterations of the cortical actin network in cells over-expressing ICA512-CCFGFP in comparison to cells over-expressing GFP (Fig. 11a). Another potential mechanism to enhance exocytosis is to increase mobility of the insulin granules so that they have a greater chance of approaching and fusing with the plasma membrane. In  $\beta$ -cells exposed to low (<5mM) glucose levels granules display mainly short oscillatory movements (<2-3 vesicle diameter), whereas stimulatory glucose concentrations (>5 mM) allow the detection of granules making excursions of several micrometers (Pouli et al., 1998), hence having a greater probability to approach the plasma membrane and fuse with it. Accordingly, we used total internal reflection fluorescence microscopy to test in living INS-1 cells whether ICA512-CCF affects the mobility of granules near ( $\leq 200$  nm) the plasma membrane. For this purpose INS-1 cells stably transfected with the granule cargo *chromogranin B* tagged with monomeric red fluorescent protein 1 (Campbell et al., 2002) (*CgB-mRFP1*) were transiently cotransfected with *GFP* (Fig. 5a, c, e) or *ICA512-CCFGFP* (Fig. 5b, d, f). Co-localization of CgB-mRFP1 with insulin granules was verified by confocal microscopy (Fig. 11b). Computer-assisted analysis with the Motiontracking II software (Rink et al., 2005), which allows automated particle tracking, revealed a density of  $28 \pm 9$  CgBmRFP1+ granules/100 $\mu$ m<sup>2</sup> in *GFP/CgB-mRFP1-INS1* cells compared to  $11 \pm 2.6$  CgBmRFP1+ granules/100  $\mu$ m<sup>2</sup> ( $p < 0.005$ ) in *ICA512-CCF-GFP/CgB-mRFP1-INS1* cells in resting conditions (Fig. 5a, b, c, d and 5g). Further, the mean speed of CgB-mRFP1+ granules in resting *ICA512-CCF-GFP/CgB-mRFP1-INS1* cells was significantly increased relative to resting *GFP/CgB-mRFP1-INS1* ( $0.113 \mu$ m sec<sup>-1</sup> vs.  $0.069 \mu$ m sec<sup>-1</sup>,  $p = 0.003$ ) (Fig. 5h). To gain further insight into the impact of ICA512-CCF on

the trajectory of cortical granules, the averaged mean square displacement of CgB-mRFP1+ vesicles was plotted against time. In *ICA512-CCF-GFP/CgB-mRFP1-INS1* cells the mean-square displacement of CgB mRFP1+ granules showed an almost linear dependency (Fig. 5i), which is characteristic of vesicles whose motion is not constrained (Huet et al., 2006). This pattern arose from frequent and random changes in the direction of the granules. Conversely, in *GFP/CgBmRFP1-INS1* cells (Fig. 5i, black trace) the mean-square displacement of CgB-mRFP1+ granules showed a linear dependency for <4 sec before curve abatement, which is characteristic of vesicles caged by the neighboring cytoskeleton network. This evidence directly proves that ICA512-CCF enhances the motility of granules by breaking their link to the cytoskeleton.

### Example 6: Materials and Methods

**Islet isolations and cell cultures.** Pancreatic islets were isolated from 15-17 weeks old *ICA512*<sup>-/-</sup> mice (Mziaut et al., 2006) and wildtype littermates as described (Gotoh et al., 1985) and then kept for 24 h in culture prior to the experiment. INS-1-derived INS-1E (Merglen et al., 2004) and tetracycline-inducible INS-r3 (Wang and Iynedjian, 1997) cell lines were gifts from C. Wollheim (University of Geneva, Geneva, Switzerland) and were grown as described (Asfari et al., 1992). Wildtype and transfected INS-1E cells and pancreatic islets were incubated for 60 minutes in resting buffer (0 mM glucose and 5mM KCl) and then for 105 minutes in fresh resting or stimulating buffer (25 mM glucose and 55 mM KCl) as described (Ort et al., 2001; Trajkovski et al., 2004). Calpeptin (Calbiochem, La Jolla, CA) was added in the last 15 minutes of the pre-incubation, and then until cells were harvested.

**cDNA constructs.** The following constructs were described: ICA512-GFP, ICA512-CCF-GFP and ICA512-HA3 (Trajkovski et al., 2004); GST-ICA512-601–979, GSTICA512-663–979, GST-ICA512-700-959, GST-ICA512-700–979, GST-ICA512extracellular and GST-ICA512-800–979 (Ort et al., 2000), ICA512-His (Ort et al., 2001), GFP- $\beta$ 2-syntrophin (Kachinsky et al., 1999). ICA512-CCF-HA3 and HA3-ICA512-CCF were generated by subcloning the cDNA encoding residues 659-979 of human ICA512 as a KpnI-XbaI insert into pMoHa3 (gift from M. Suchanek and C. Thiele, Max Planck Institute of Cell Biology and Genetics, Dresden, Germany) or as a

BglII-EcoR1 insert into pN3HA (Clontech, Palo Alto, CA), respectively. For tetracycline inducible expression of ICA512-CCF-GFP, the corresponding cDNA (Trajkovski et al., 2004) was subcloned as a BamHI/XbaI insert between the tet-operator-tk-minimal promoter and the SV40 polyA in a vector derived from the tet-tk-luc plasmid (Anastassiadis et al., 2002). Subsequently the tet-tk-ICA512-CCF-GFP-pA cassette was cloned between chicken  $\beta$ -globin insulators in a vector kindly provided by K Anastassiadis (Biotec TUD, Dresden, Germany). The mouse cDNA of the granule cargo chromogranin B (CgB; gift from W. Huttner, Max Planck Institute of Cell Biology and Genetics, Dresden, Germany) was fused in frame at the 5'-end of monomeric Red Fluorescent Protein (mRFP1) cDNA (Planetgene, Menlo Park, CA). All clones were generated using conventional procedures and verified by DNA sequencing.

**Cell Transfections.** INS-1E cells were electroporated as described (Trajkovski et al., 2004). The generation of stable GFP- and ICA512-CCF-GFP INS-1 cell clones was described (Trajkovski et al., 2004). INS-r3 cells expressing the reverse tetracyclinedependent transactivator were transiently transfected with ICA512-CCF-GFP in the tettk-luc-derived plasmid. The expression of ICA512-CCF-GFP was induced by exposure to 500ng/ml doxycyclin for 16 and 40 hours. CgB-mRFP1 INS-1 clones were selected for puromycin resistance, and further screened by fluorescence microscopy and immunoblotting for expression of the transgene.

**Cell extracts and western blottings.** Purified mouse islets and INS-1E cells were washed with ice cold PBS, and extracted in lysis buffer (10 mM Tris-HCl at pH 8.0, 140 mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM PMSF, 1% phosphatase inhibitors (Calbiochem), and 1% protease inhibitor cocktail (Sigma, St. Louis, MO) at 4 °C. Protein measurements and immunoblottings were performed as described (Trajkovski et al., 2004) using the following primary antibodies: mouse monoclonals antibodies mICA512 (Hermel et al., 1999), mICA512-HM1 (Mziaut et al., 2006), anti- $\beta$ -tubulin (Sigma) and anti-GFP (Clontech); rabbit polyclonal anti-glut2 (Alpha Diagnostics International, St Antonio, TX), anti-ICA512ecto (Solimena et al., 1996) anti-carboxypeptidase E/H (CPE) (Chemicon, Temecula, CA), goat anti-GFP (gift from David Drechsel, Max Planck Institute of Cell Biology and Genetics, Dresden,

Germany). Chemiluminescence was developed and quantified as described (Trajkovski et al., 2004).

**Sucrose density gradient fractionations.** Fractionations of INS-1E cells on continuous sucrose density gradients from 0.4-2 M sucrose were performed as described (Iezzi et al., 1999; Solimena et al., 1996). Fractions were separated by SDS-PAGE and analyzed by immunoblotting.

**Immunoprecipitations.** INS-1E cells were transiently or stably transfected with one or more of the following constructs: *GFP*, *ICA512-CCF-GFP*, *GFP-β2-syntrophin*, *ICA512-CCF-HA3*, *HA3-ICA512-CCF*. Protein extracts in 20 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM EDTA, 1mM PMSF, 1% phosphatase inhibitors (Calbiochem) and 1% protease inhibitor cocktail (Sigma) were incubated overnight at 4 °C with one of the following antibodies: goat anti-GFP, rabbit anti ICA512ecto, goat or rabbit IgGs (Sigma), followed by incubation with protein G sepharose (GE Healthcare Biosciences, Uppsala, Sweden) for 90 minutes. The beads were then washed 10 times, and loaded on 8-10% SDS-PAGE followed by western blotting with one of the following mouse antibodies: anti-ICA512, anti-GFP (Clontech, Mountain View, CA), anti- β2-syntrophin (a gift from S. Froehner and M. Adams, University of Washington, Seattle), anti-HA and anti-γ-tubulin (Sigma).

**Pulldown Assays.** ICA512601–979, ICA512700-979 or β2-syntrophin in pET28a (Ort et al., 2000) were in vitro transcribed and translated with the T7/coupled transcriptiontranslation system (Promega, Madison, WI). GST-ICA512-601–979, GST-ICA512-663–979, GST-ICA512-700-959, GST-ICA512-700–979, GST-ICA512extracellular, GST-ICA512-800–979 and ICA512-His were expressed in bacteria as described (Mziaut et al., 2006; Ort et al., 2001). <sup>35</sup>S-methionine-labeled ICA512-601–979, ICA512-700-979 or β2-syntrophin were incubated overnight at 4 °C in GST binding buffer (240 mM NaCl, 50 mM Tris, pH 8, 0.25% NP-40, 0.15% SDS, 1 mM DTT, and 1 mM PMSF) with GST-fusion proteins or GST alone coupled to glutathione-sepharose beads (Amersham Biosciences). Beads were washed 10 times with binding buffer and eluted with 10 mM of reduced glutathione. Eluted proteins were subjected to SDS-PAGE and analyzed by autoradiography.

**Immunocytochemistry.** Labeling of transfected or non-transfected INS-1E cells was performed as described (Trajkovski et al., 2004). The following primary and secondary antibodies were used: mouse anti-insulin (Sigma), rabbit anti-TGN38 (BD Transduction Laboratories, San Jose, CA), Alexa568-conjugated goat-anti-mouse or Alexa488- conjugated-goat-anti-rabbit IgGs (Molecular Probes, Eugene, OR) and phalloidin-rhodamine (Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI (Sigma). Coverslips were mounted with Mowiol (Calbiochem). Images of 0.5  $\mu$ m optical sections were acquired with an inverted confocal microscope LSM Meta 405nm (Zeiss, Göttingen, Germany) or with an inverted confocal microscope Zeiss Axiovert 200M equipped with a Plan-Apochromat 63X oil objective, numerical aperture 1.4, a Zeiss LSM510 scanhead with photo-multiplier tubes and the software Zeiss LSM 510 AIM V.4 (Zeiss, Göttingen, Germany).

**RNA interference.** For knockdown of rat ICA512 and  $\beta$ 2-syntrophin the pGENE-CLIP U1 cassette system (Promega) was used according to the manufacturer's instruction to clone the following oligonucleotides: ICA512 shRNA oligo1, 5'-TCTCGCGCCATCATTGAAACAATTCAAGAGATTGTTTCGAATGATGGCGCCT-3'; ICA512 shRNA oligo2, 5'-TCTCGCAGTACAAGCAGATGTAATTCAAGAGATTACATCTGCTTGTACTGCCT-3';  $\beta$ 2-syntrophin shRNA oligo1: 5'-TCTCGTAGCTATCCACACCAACATATTCAAGAGATATGTTGGTGTGGATAGCTACCT-3'; CTGCAGGTAGCTATCCACACCAACATATCTCTTGAATATGTTGGTGTGGATAGCTAC-3'. Single or double transfections of INS-1E cells with pGENE-ICA512 and pGENE- $\beta$ 2-syntrophin by electroporation were performed as described (Trajkovski et al., 2004), using 4 $\mu$ g or 3 $\mu$ g of each plasmid for single or double transfection, respectively. Cells were stimulated and harvested 4 days after transfection and knockdown of ICA512 and  $\beta$ 2-syntrophin was determined by real-time PCR and western blotting, as described Trajkovski et al., 2004).

**Insulin RIA.** Insulin secretion was assessed as a ratio between insulin in the medium and the cell insulin content. After stimulation, 25-30 purified pancreatic islets were resuspended in fresh resting or stimulating medium and sonicated for 4 minutes. INS-1E cells were extracted overnight in 3:1:0.06 volumes of ethanol, H<sub>2</sub>O and 37%

HCl at  $-20^{\circ}\text{C}$ . Following centrifugation, insulin in islet cells and in the medium was measured with the Sensitive Rat Insulin RIA Kit (Linco Research, St. Charles, MO). The insulin stimulation index was calculated as follows: secreted/total insulin in stimulated conditions vs. secreted/total insulin in resting conditions.

**TIRFM.** Images of INS-1 cells stably expressing mouse CgB-mRFP1 were acquired with a Roper Scientific MicroMAX:512BFT charge-coupled device camera with a Zeiss Axiovert 200M microscope equipped with an Argon laser and a Zeiss 100x/1.45 N.A. Plan-FLUAR objective and a 1.6X optovar. The microscope was outfitted with a dualport TIRF condenser (Till Photonics, Gräfelfing, Germany). Prior to imaging, cells grown in an open chamber were incubated in resting media and transferred onto a thermostat-controlled ( $37^{\circ}\text{C}$ ) stage. CgB-mRFP1+ granules in GFP+ cells were visualized by excitation of mRFP1 at 488 nm using filter sets for tetramethylrhodamine isothiocyanate emission (Chroma Technology Corp, Rockingham, VT). Images were collected at the speed of 2 frames second<sup>-1</sup> with an exposure time of 100 ms, for a total of 4 minutes (480 frames) each movie. Automated image analysis was performed with the Motiontracking II software as described (Rink et al., 2005). The background of nonvesicular fluorescence was subtracted and the granules were fitted by analytical function as described (Rink et al., 2005). The accuracy of granule center definition was estimated from the mean square displacement, i.e. the average of squared displacements, and found equal to 60 nm. The jitter of center definition was suppressed by smoothing the tracks with sliding window ( $\pm 2$  frames). The total number of tracked CgB-mRFP1+ granules in GFP+ and ICA512-CCF-GFP+ cells was 779 and 888, respectively. The speed was measured between every two sequential frames. The total number of speed measurements in GFP+ and ICA512-CCF-GFP+ cells was 13,332 and 14,828 respectively.

**Statistics and graphics.** Statistical analyses were performed as previously described (Mziaut et al., 2006; Trajkovski et al., 2004). Error bars show standard deviations from at least three independent experiments. Histograms were prepared with Microsoft Excel (Microsoft, Redmond, WA).

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### Claims

1. A pharmaceutical composition comprising
  - (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or a mimetic of the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;
  - (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN, wherein the removed C-terminal fragment includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
  - (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
  - (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.
2. Use of
  - (a) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin, or of a mimetic of the C-terminus of ICA512,

pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;

- (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, wherein the C-terminal fragment removed includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
- (c) a compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (d) a compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
- (e) a compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
- (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110

for the preparation of a pharmaceutical composition for the treatment of diabetes.

3. The pharmaceutical composition of claim 1 or the use of claim 2 wherein said mimetic is a peptidomimetic.
4. The pharmaceutical composition of claim 1 or the use of claim 2 wherein the compound promoting the presence or activity of said protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN is an expression vector containing the coding region of said protease in expressible form.
5. The pharmaceutical composition of claim 1 or the use of claim 2 wherein the compound promoting the presence or activity of said serine/threonine kinase is an expression vector containing the coding region of said kinase in expressible form.

6. The pharmaceutical composition of claim 1 or the use of claim 2 wherein the compound promoting the presence or activity of said serine/threonine phosphatase is an expression vector containing the coding region of said phosphatase in expressible form.
7. A method of enhancing insulin secretion from pancreatic  $\beta$ -cells comprising promoting in said  $\beta$ -cells the presence or activity of
  - (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;
  - (b) a compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, wherein the C-terminal fragment removed includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
  - (c) a compound promoting the presence or activity of serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (d) a compound interfering with the presence or activity of serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (e) a compound promoting the presence or activity of a phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
  - (f) a compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.
8. The method of claim 7 wherein the promotion comprises the stable or transient expression from an exogenously introduced vector of said

- (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;
  - (b) compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, wherein the C-terminal fragment removed includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
  - (c) compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (d) compound interfering with the expression or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (e) compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or
  - (f) compound interfering with the expression or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.
9. The pharmaceutical composition of any one of claims 1 to 6, the use of any one of claims 2 to 6 or the method of claim 7 wherein the promotion comprises reducing degradation or enhancing stability in said  $\beta$ -cells of said
- (a) (aa) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN;  
or  
(ab) ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF, each devoid of the C-terminus or PHOGRIN devoid of the C-terminus, wherein said ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or

PHOGRIN does not comprise a functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin;

- (b) compound promoting the presence or activity of a protease that cleaves the C-terminus of ICA512, pro ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN, wherein the C-terminal fragment removed includes the functional domain involved in the binding to the PDZ domain of  $\beta$ 2-syntrophin or a portion of said functional domain wherein said portion is sufficient to hinder binding of ICA512, pro-ICA512, ICA512-TMF or ICA512-CCF or PHOGRIN to  $\beta$ 2-syntrophin;
  - (c) compound promoting the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (d) compound interfering with the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 95;
  - (e) compound promoting the presence or activity of a serine/threonine phosphatase which dephosphorylates  $\beta$ 2-syntrophin on serine residue 110; or said
  - (f) compound interfering with the presence or activity of a serine/threonine kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 110.
10. The pharmaceutical composition of any one of claims 1 to 6 and 9, the use of any one of claims 2 to 6 and 9 or the method of any one of claims 7 to 9 wherein the compound interfering with the presence or activity of said phosphatase or of said kinase is an siRNA, an shRNA, a ribozyme, a peptide aptamer, a nucleic acid based aptamer, a small molecule or an antibody.
11. A method of identifying a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells comprising contacting a compound or a plurality of compounds suspected to be useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells with a mixture of (a) ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or

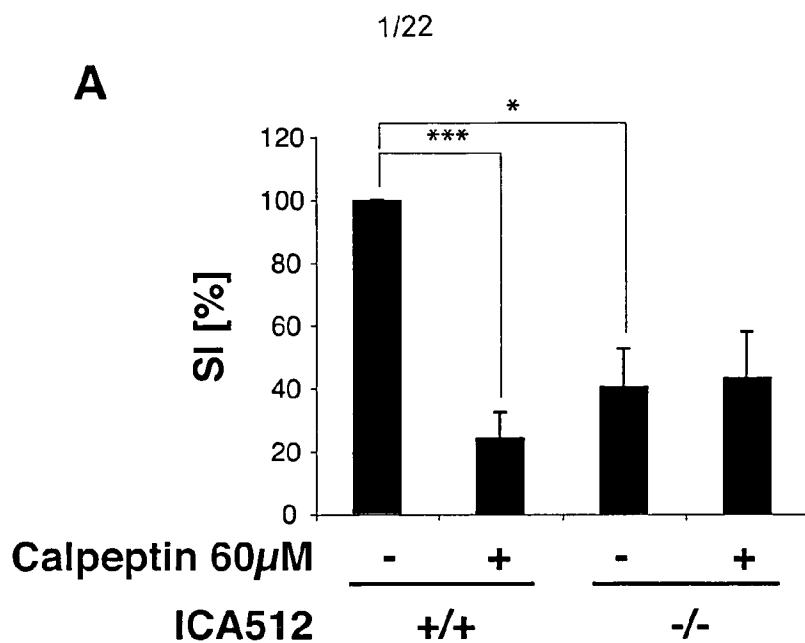
PHOGRIN; and (b)  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin *in vitro* under conditions that would allow binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin in the absence of the compound or the plurality of compounds and assessing binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin wherein an interference of binding of ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF and/or PHOGRIN to  $\beta$ 2-syntrophin and/or the PDZ domain of  $\beta$ 2-syntrophin in the presence of said compound or said plurality of compounds is indicative of said compound or at least one member of said plurality of compounds being useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful in the enhancement of insulin secretion from pancreatic  $\beta$ -cells.

12. A method of identifying a compound useful in the treatment of diabetes or useful as a lead compound for developing a compound useful in the treatment of diabetes comprising *in silico* assessing a crystal of ICA512 or PHOGRIN or a crystal of a fragment of ICA512 or PHOGRIN comprising the PTP domain for a molecular structure fitting into the binding pocket of the PTP domain.
13. A method of identifying a compound useful for enhancing insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful enhancing insulin secretion from pancreatic  $\beta$ -cells comprising *in silico* assessing a crystal of ICA512 or PHOGRIN or a crystal of a fragment of ICA512 or PHOGRIN comprising the PTP domain for a molecular structure fitting into the binding pocket of the PTP domain.
14. A method of identifying a compound useful for enhancing insulin secretion from pancreatic  $\beta$ -cells or useful as a lead compound for developing a compound useful enhancing insulin secretion from pancreatic  $\beta$ -cells comprising *in silico* assessing a crystal of  $\beta$ 2-syntrophin or a crystal of a fragment of  $\beta$ 2-syntrophin comprising the PDZ domain for a molecular structure fitting into the binding pocket of the PDZ domain.

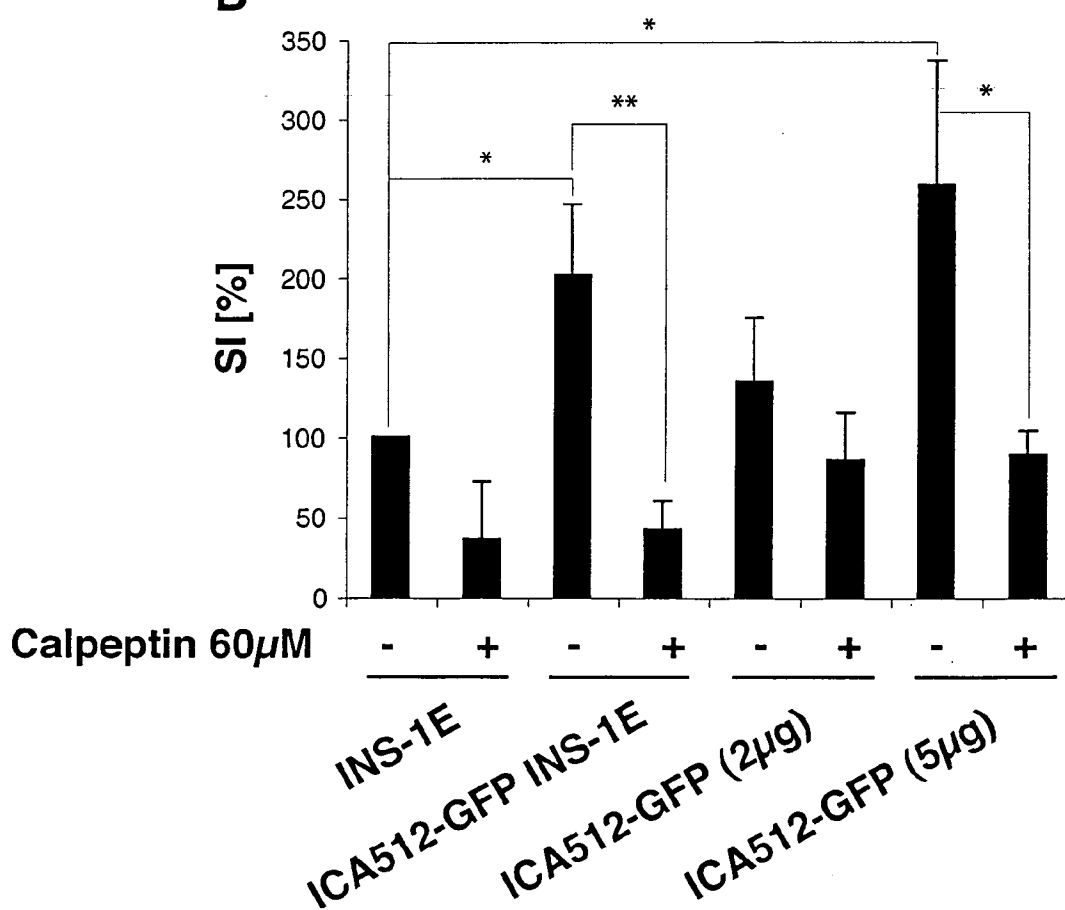
15. The method of any one of claims 12 to 14 further comprising *in silico* or *in vitro* assessing whether (a) the compound fitting into the binding pocket of the PTP domain interferes with binding of said domain or of ICA512, pro-ICA512, ICA-TMF, ICA-CCF or PHOGRIN with  $\beta$ 2-syntrophin; or (b) the compound fitting into the binding pocket of the PDZ domain interferes with binding of said domain or of  $\beta$ 2-syntrophin with ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN.
16. The method of claim 15 further comprising chemically synthesizing (a) the compound fitting into the binding pocket of the PTP domain and *in silico* or *in vitro* interfering with the binding of the PTP domain or of ICA512, pro-ICA512, ICA-TMF, ICA-CCF or PHOGRIN to  $\beta$ 2-syntrophin; or (b) the compound fitting into the binding pocket of the PDZ domain and *in silico* or *in vitro* interfering with the binding of the PDZ domain or  $\beta$ 2-syntrophin with ICA512, pro-ICA512, ICA512-TMF, ICA512-CCF or PHOGRIN.
17. The pharmaceutical composition of any one of claims 1 to 6, the use of any one of claims 1 to 6 or the method of any one of claims 7 to 16 wherein said kinase which phosphorylates  $\beta$ 2-syntrophin on serine residue 95 is CDK5.
18. The pharmaceutical composition, use or method of any of the preceding claims wherein said compound is a small molecule, a peptide, and antibody, a peptide aptamer, a nucleic acid aptamer, a glucose metabolite, an anticalin, an siRNA, an shRNA, a ribozyme, an antisense nucleic acid molecule or a mimetic, preferably a peptidomimetic.
19. Use of a glucose metabolite or a derivative thereof for the preparation of a pharmaceutical composition for the treatment of diabetes.
20. The use of claim 19 wherein said glucose metabolite is glyceraldehyde-3-phosphate.
21. The pharmaceutical composition, use or method of any of the preceding claims wherein said diabetes is type-2 diabetes.

Figure 1

A

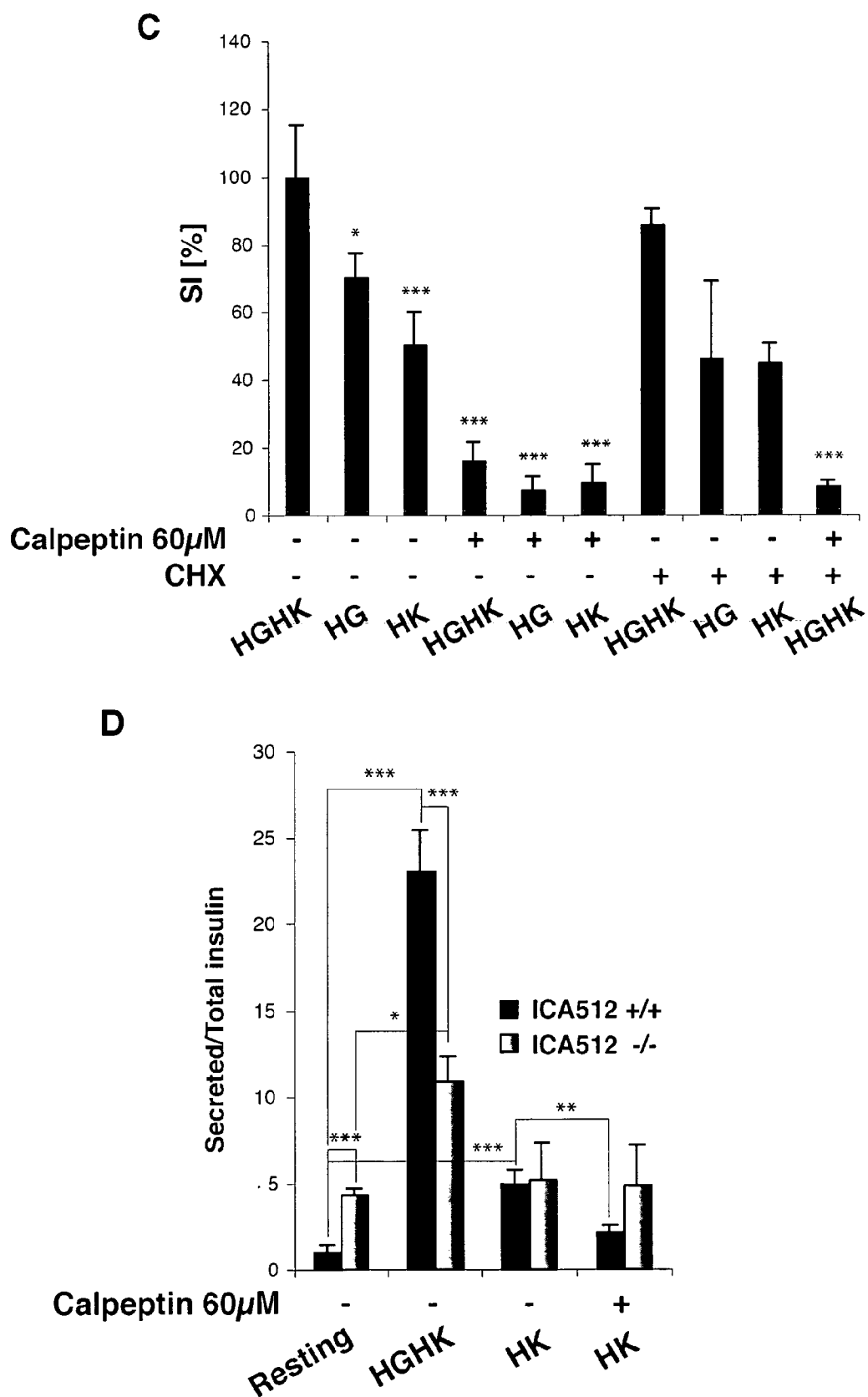


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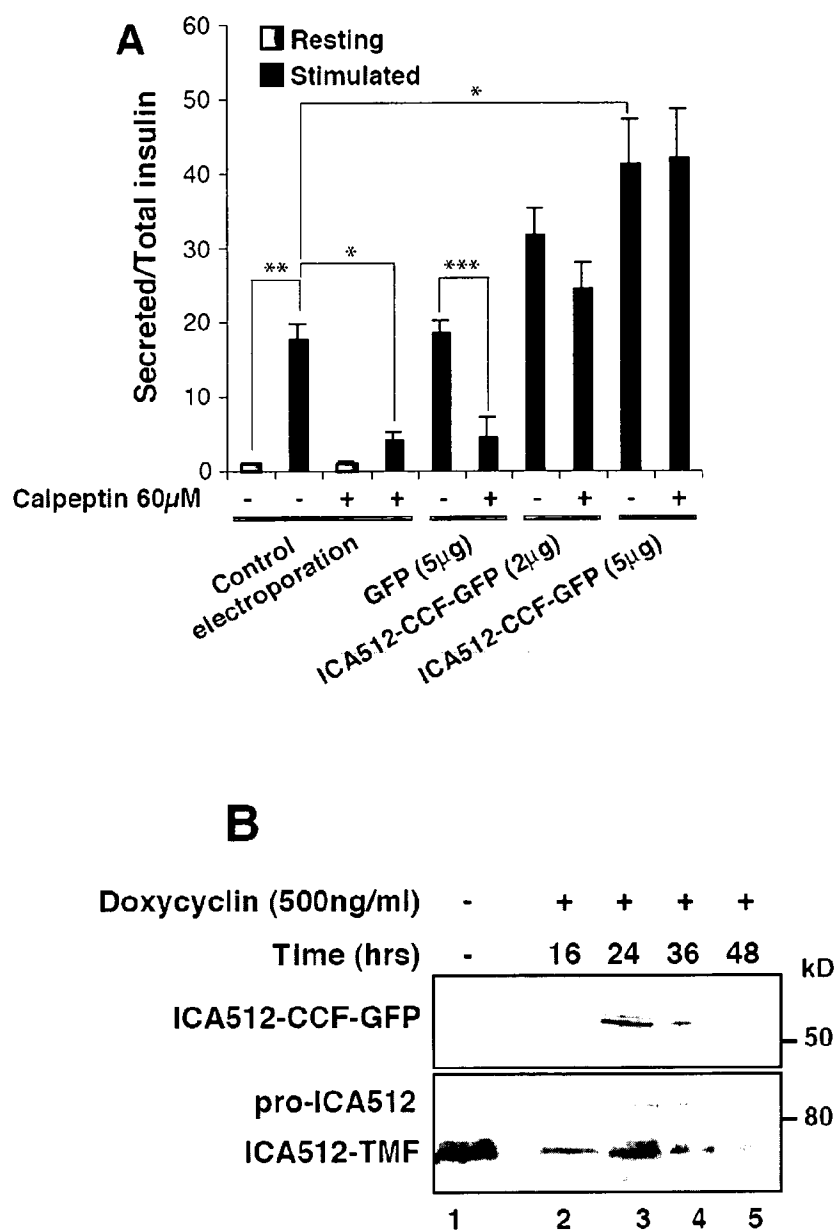
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Figure 1



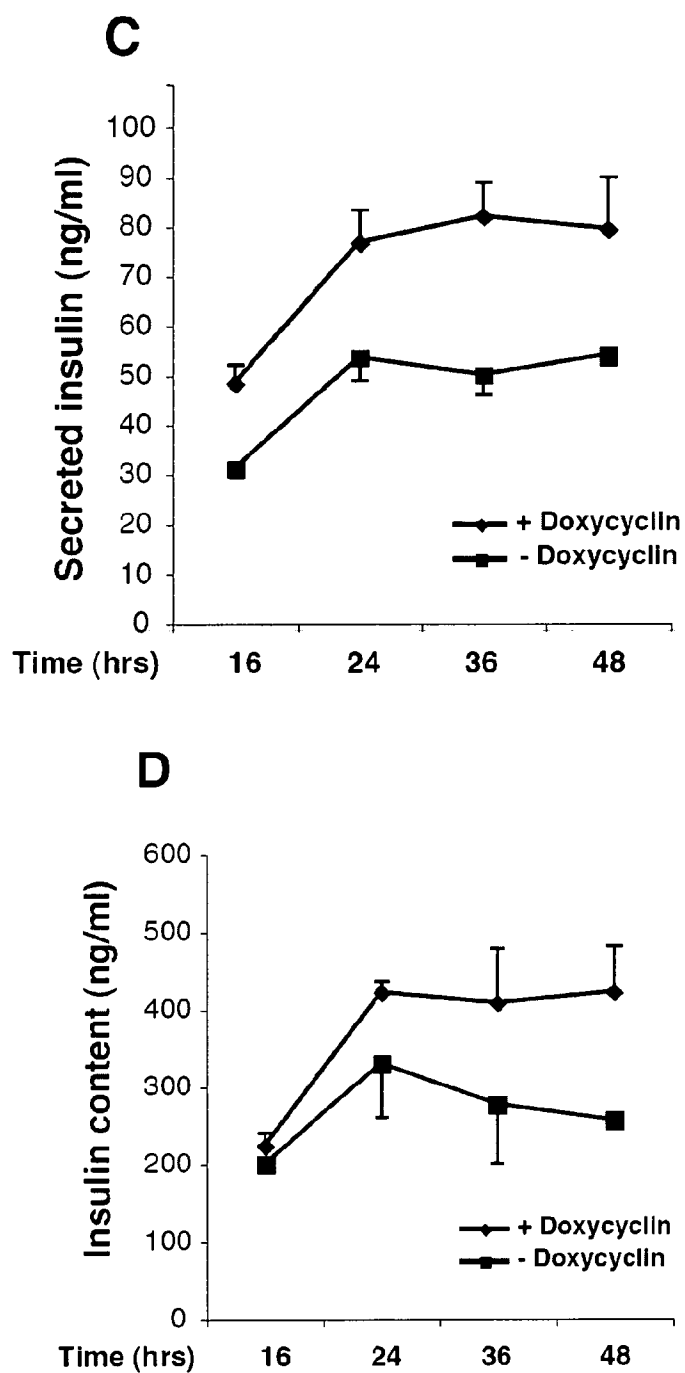
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Figure 2



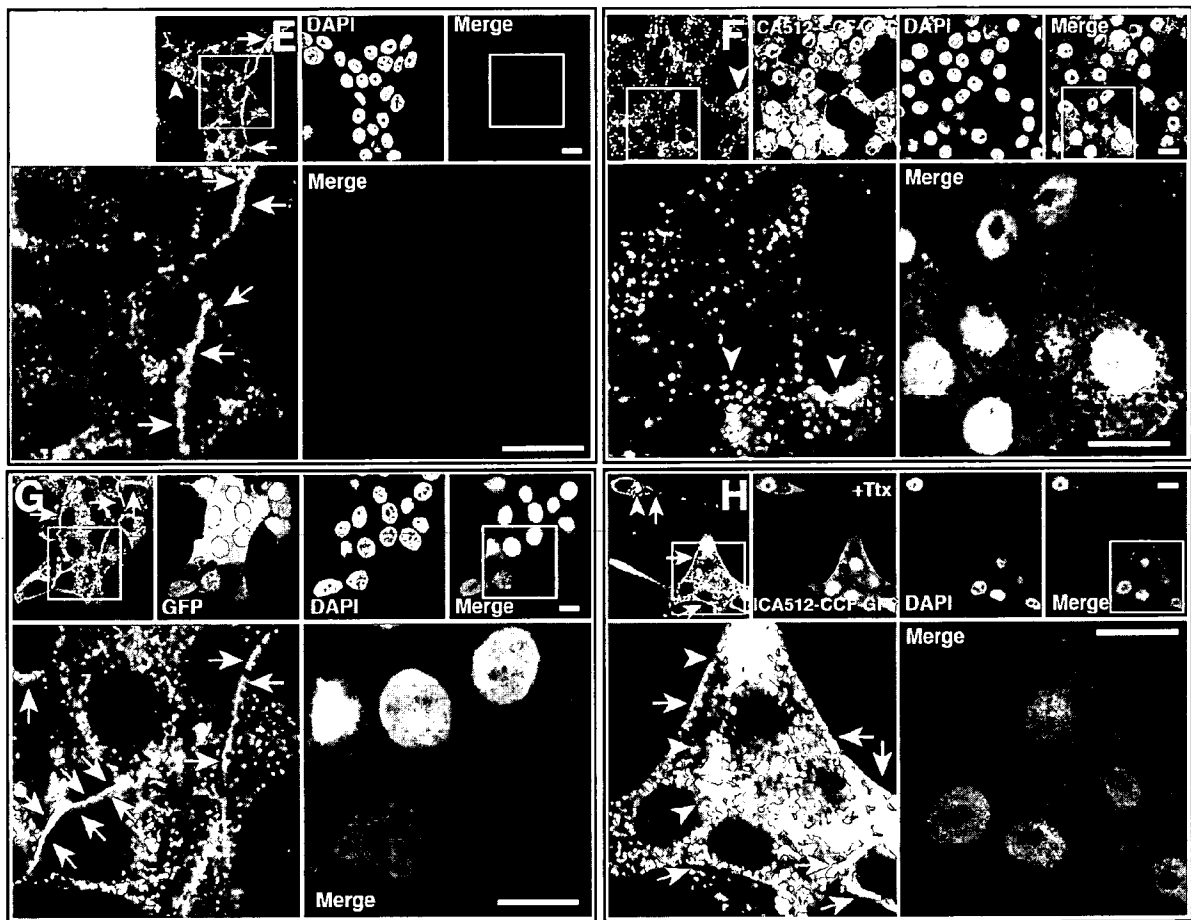
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Figure 2



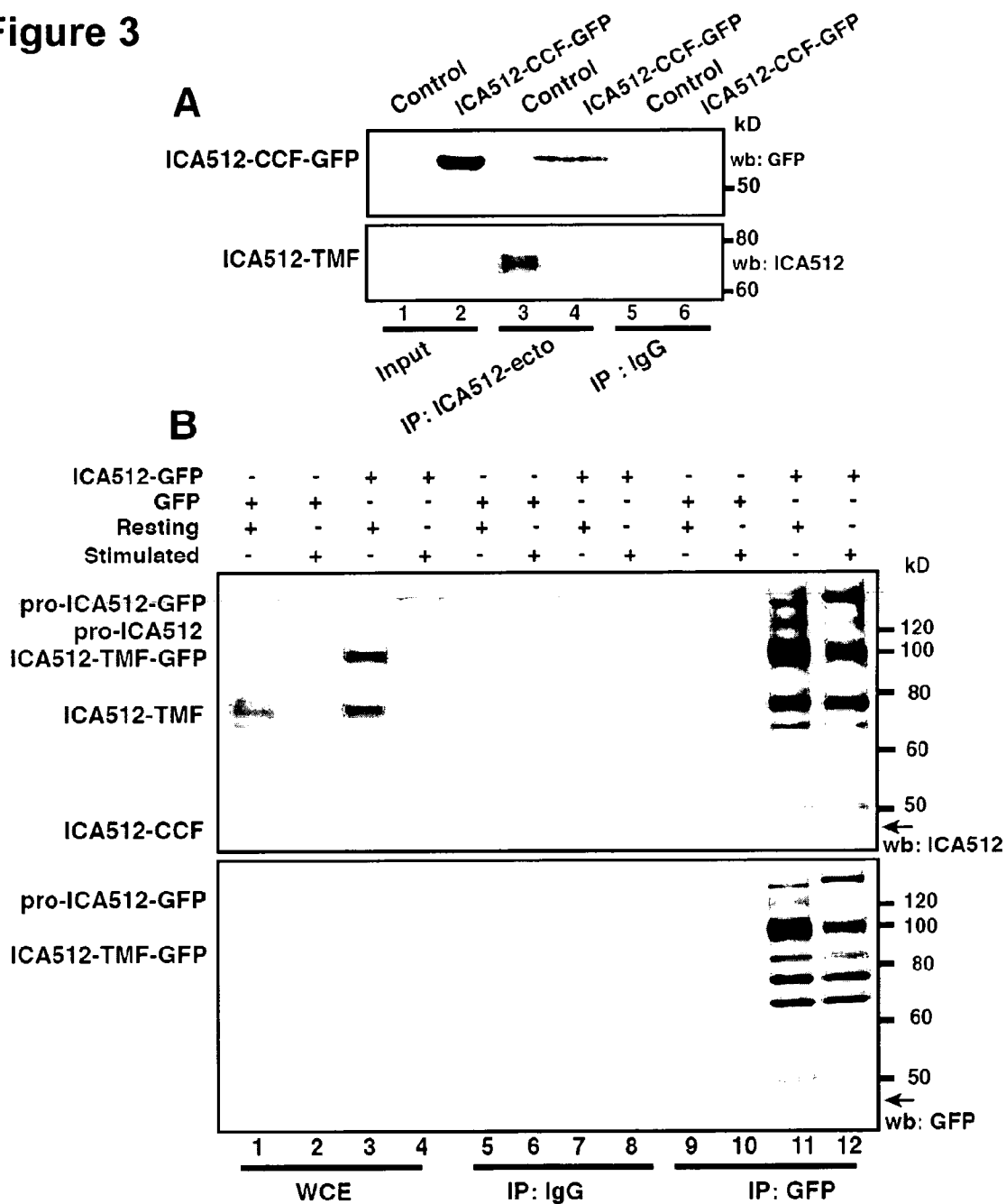
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Figure 2



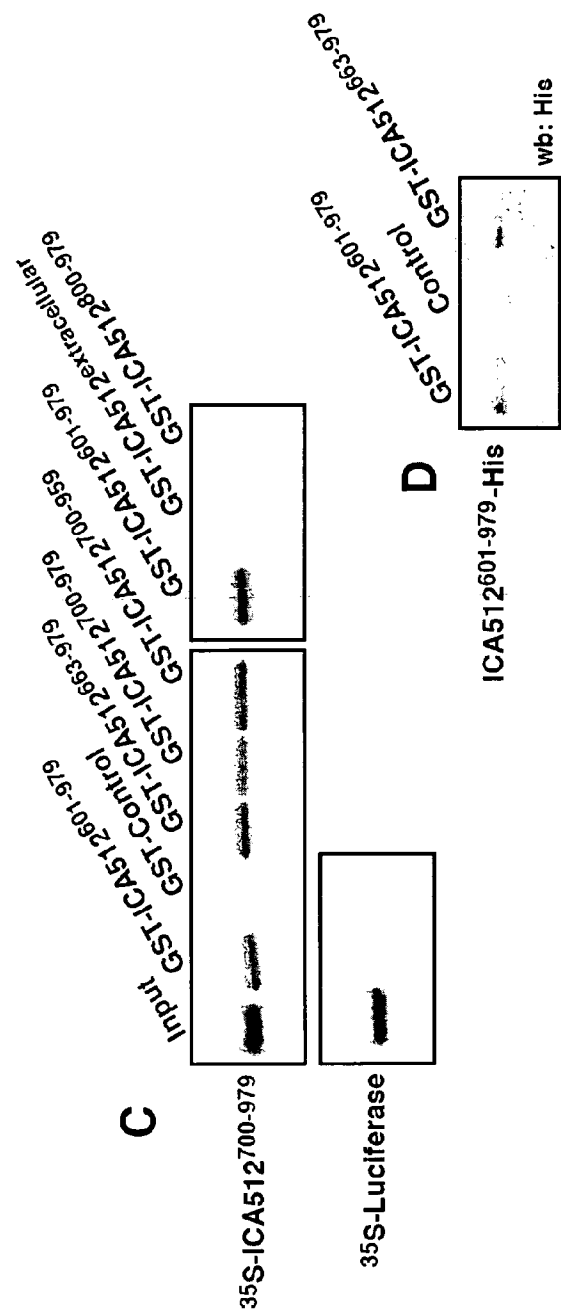
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**Figure 3**

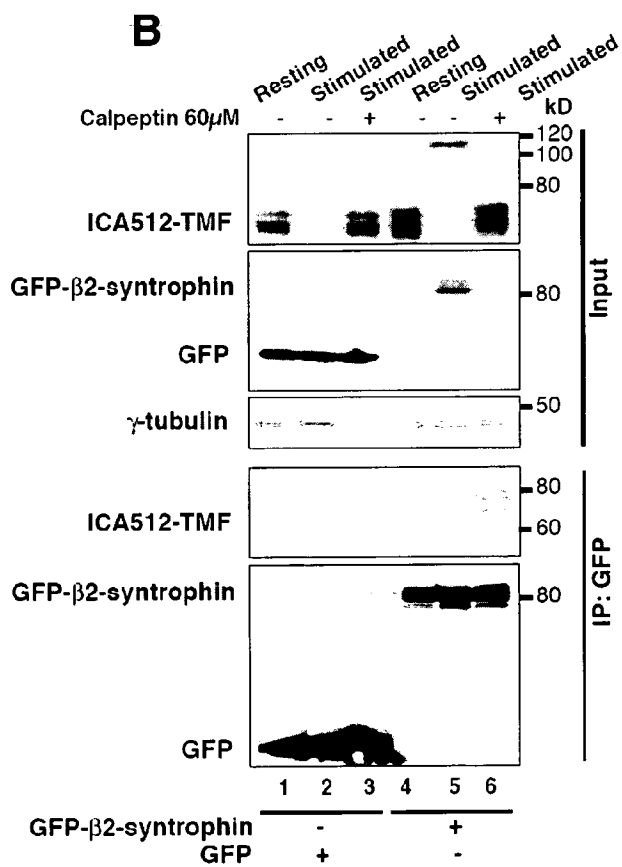
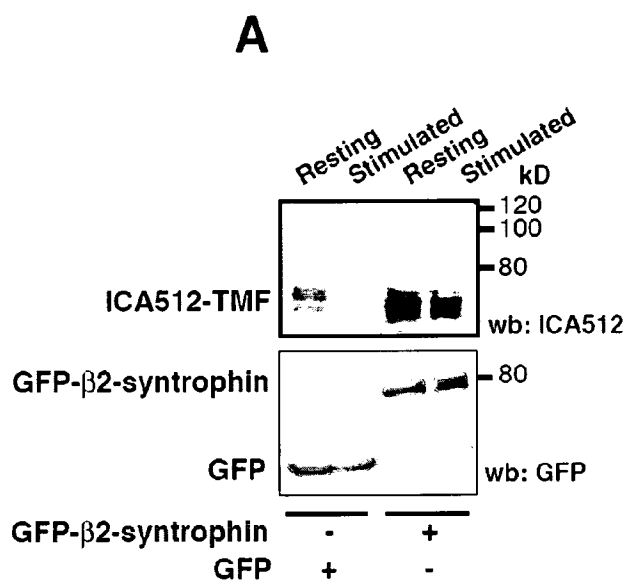


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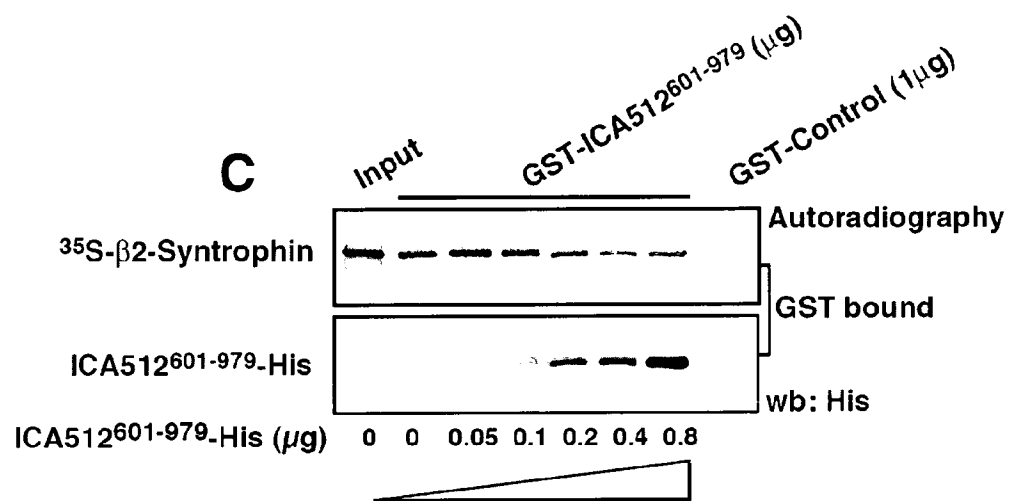
Figure 3



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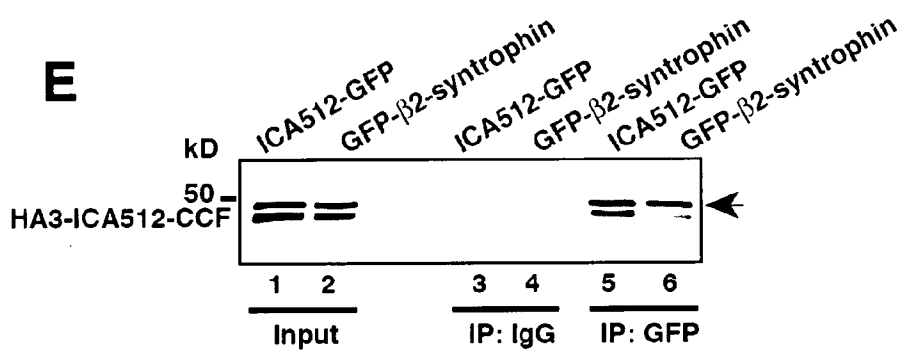
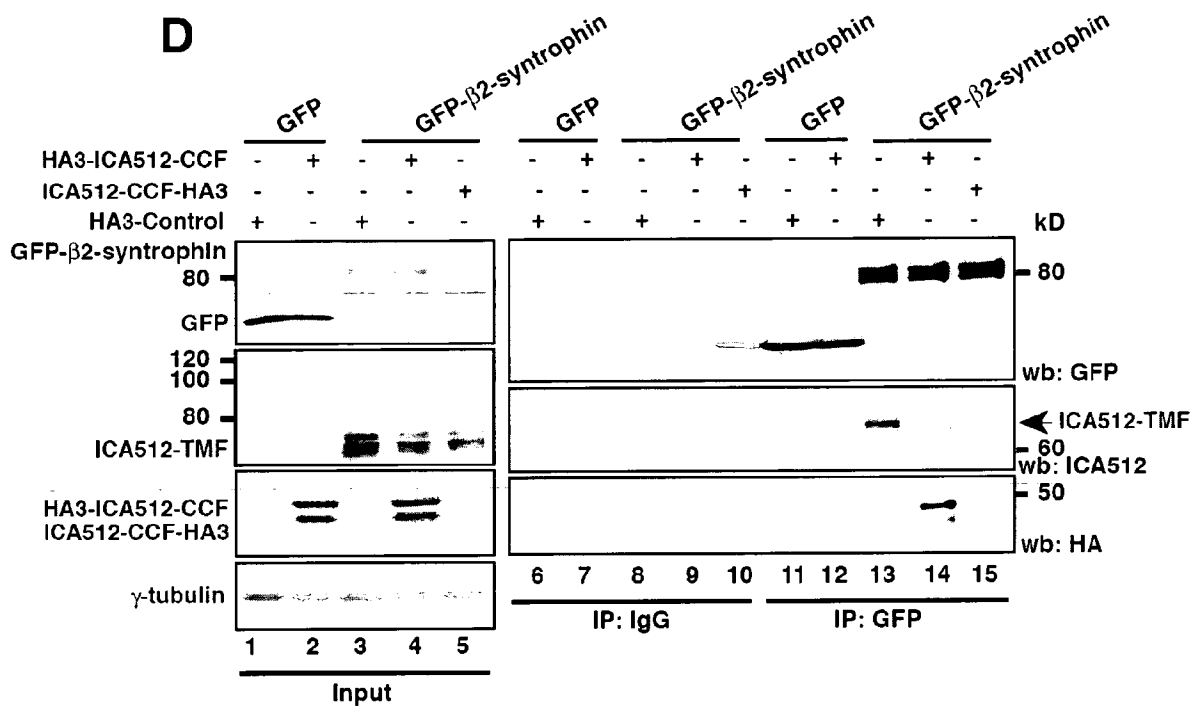
**Figure 4**

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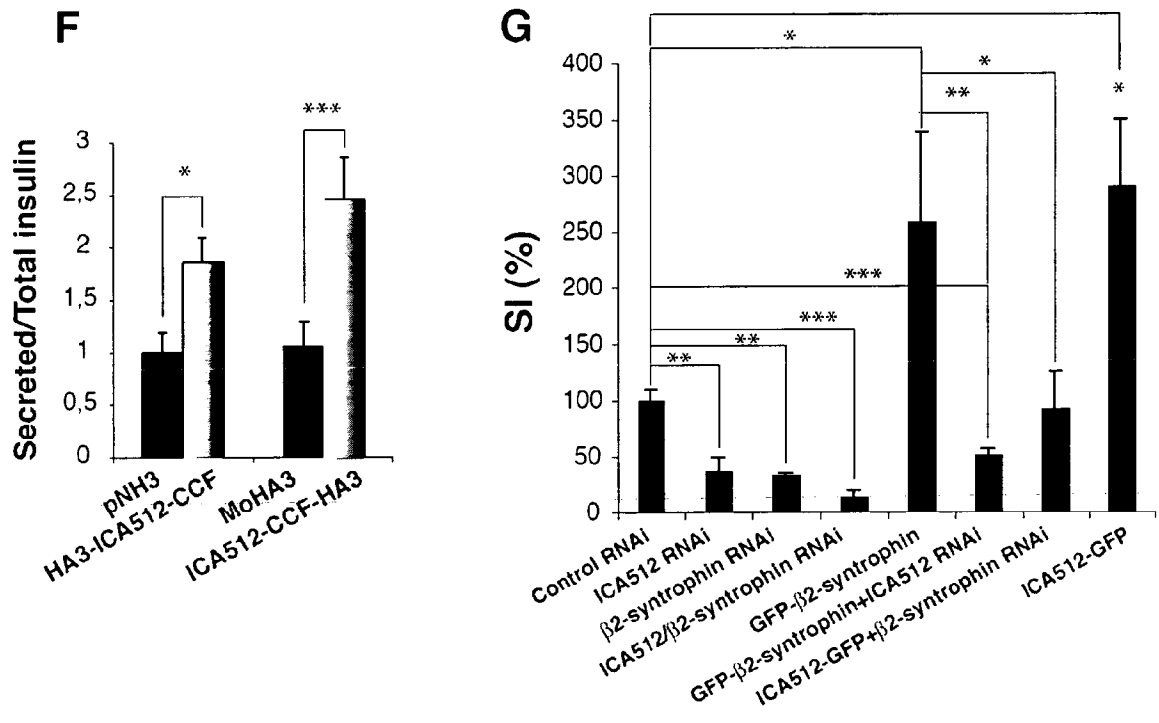
**Figure 4**

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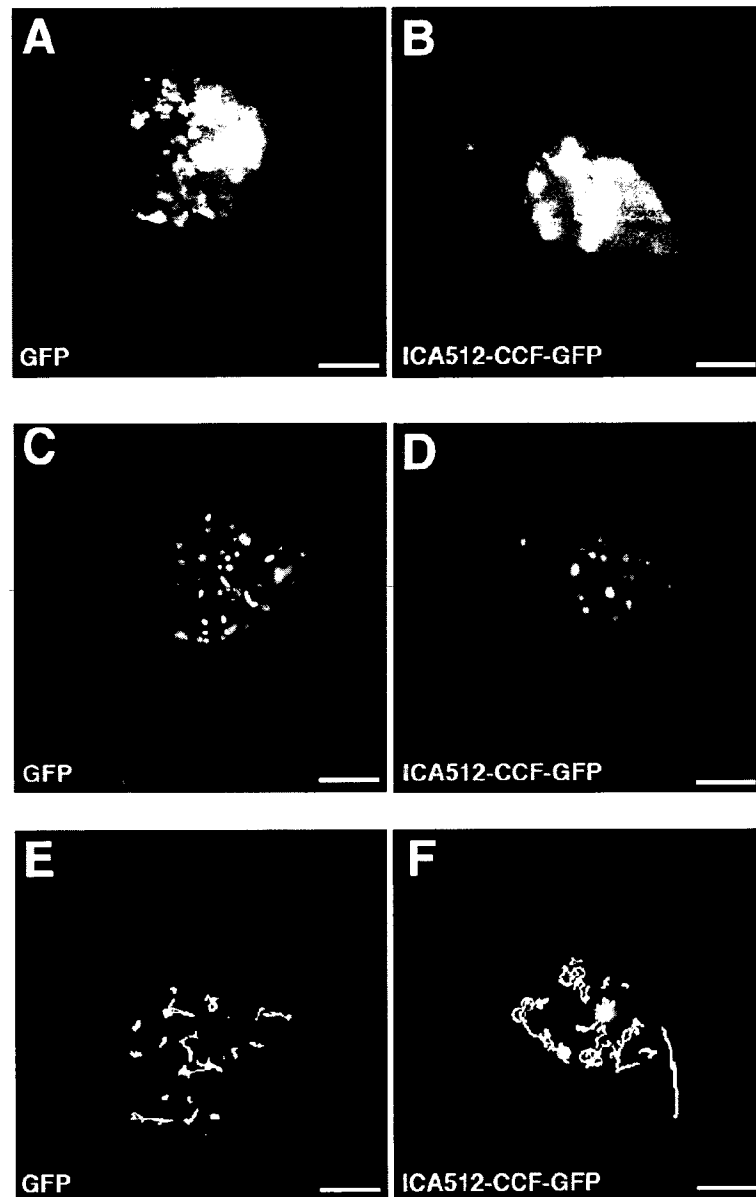
Figure 4



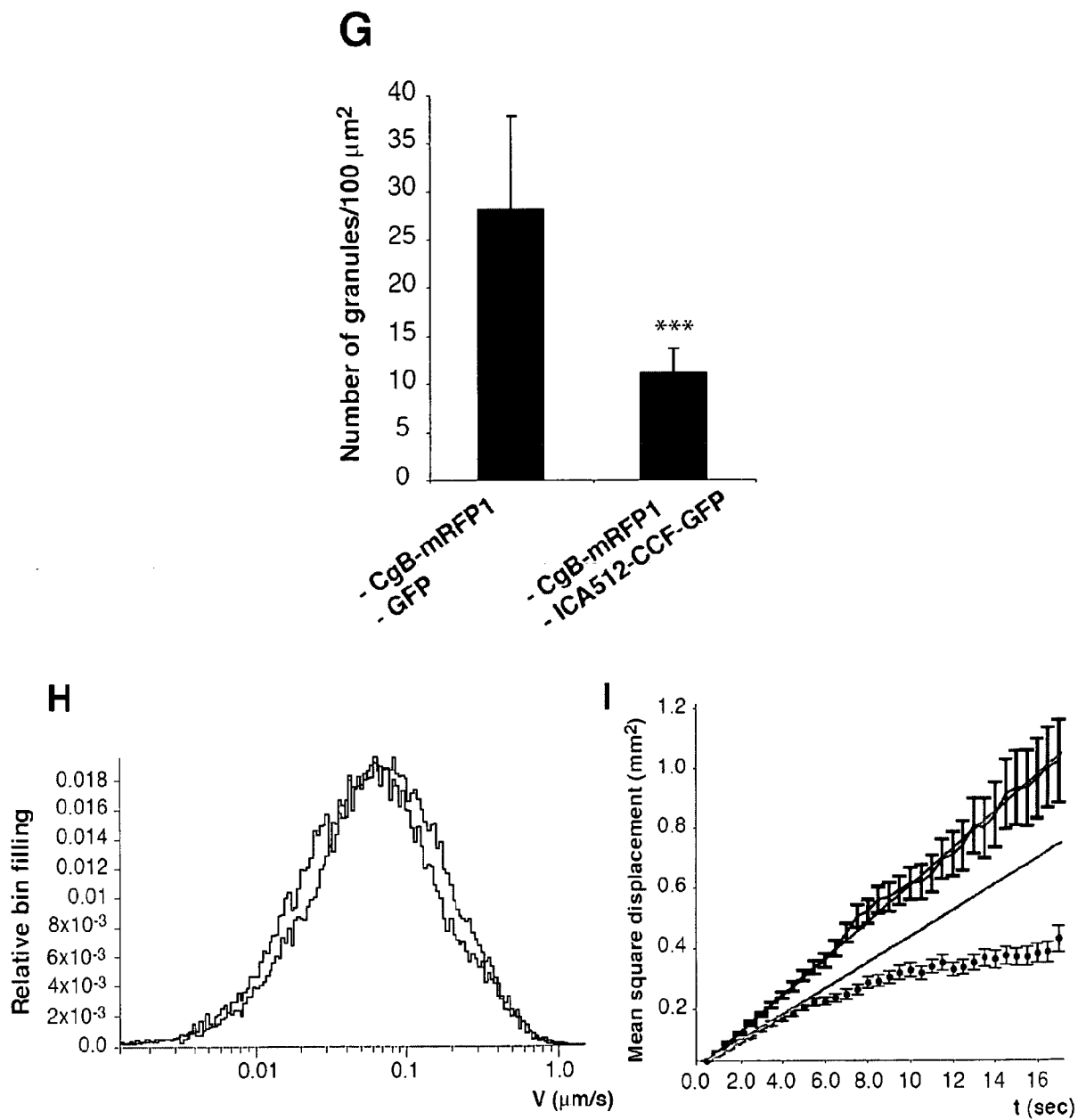
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**Figure 4**

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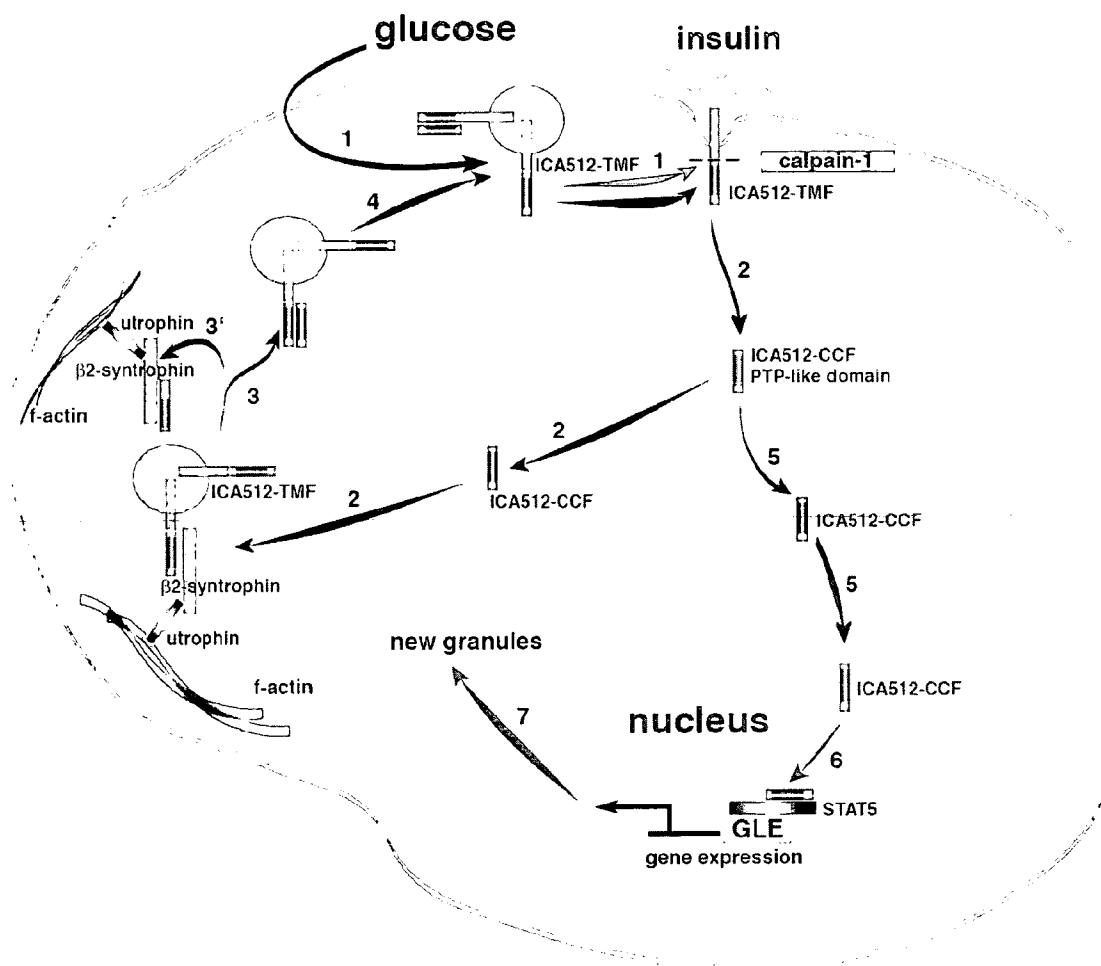
**Figure 5**

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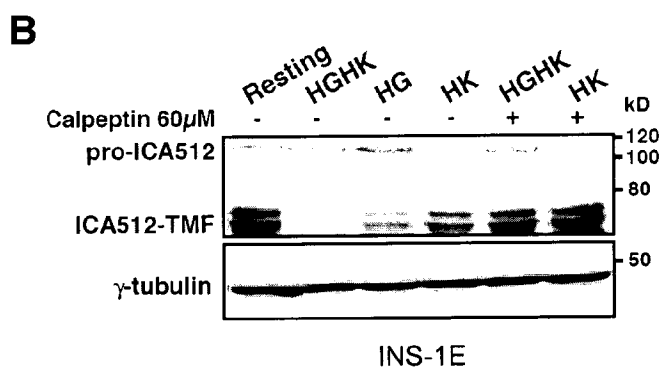
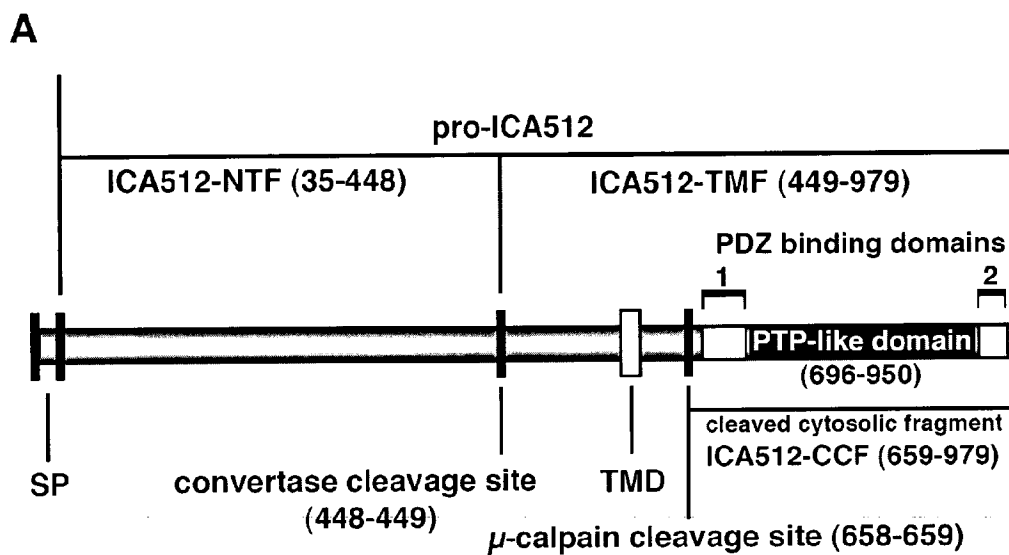
**Figure 5**

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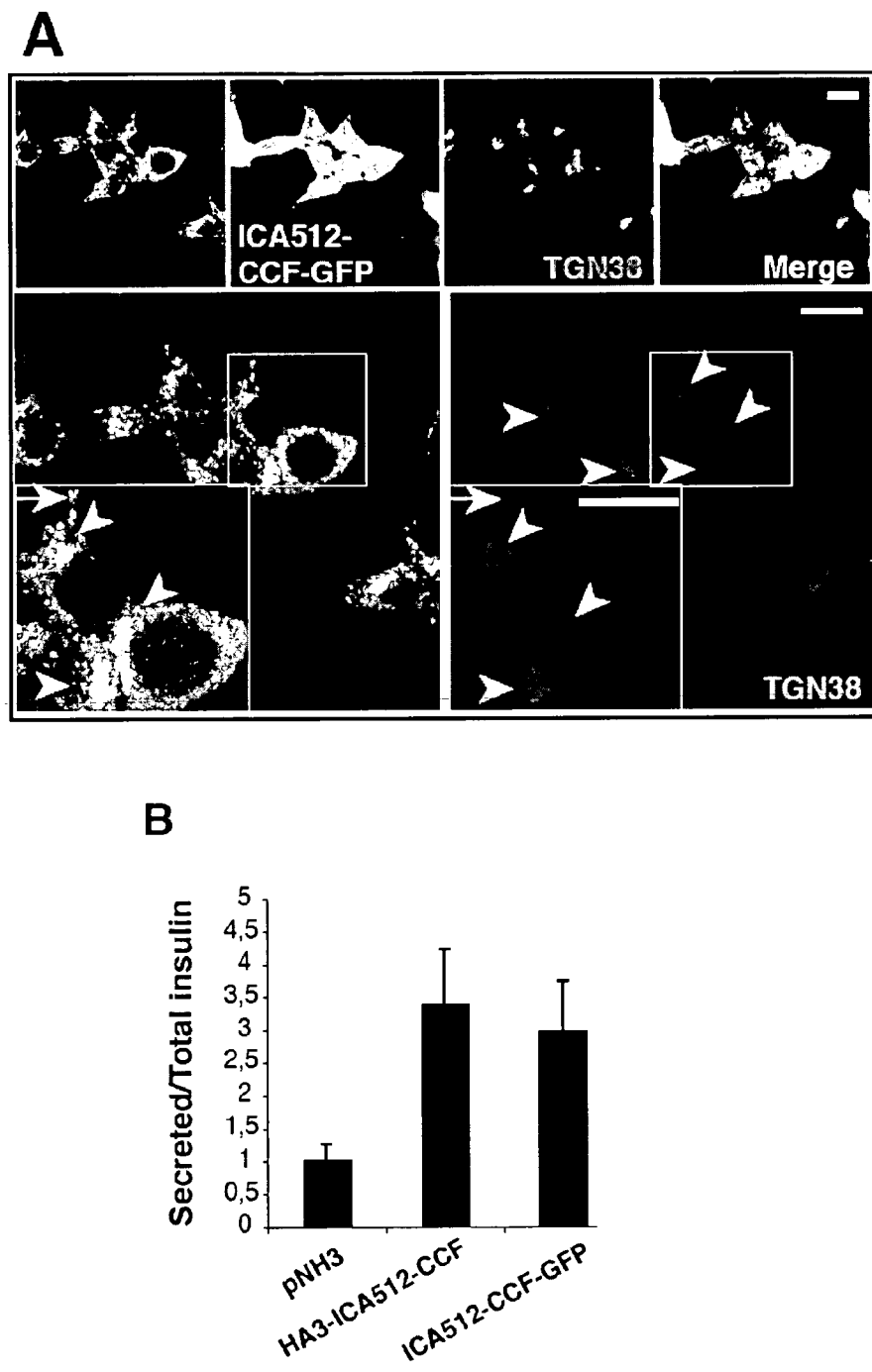
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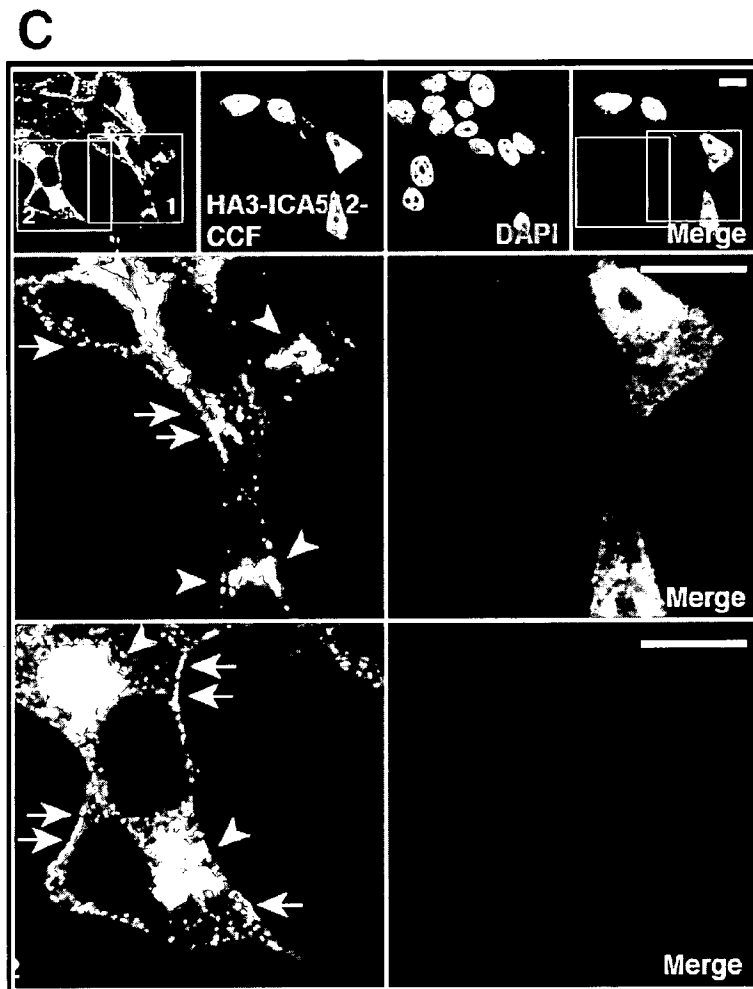


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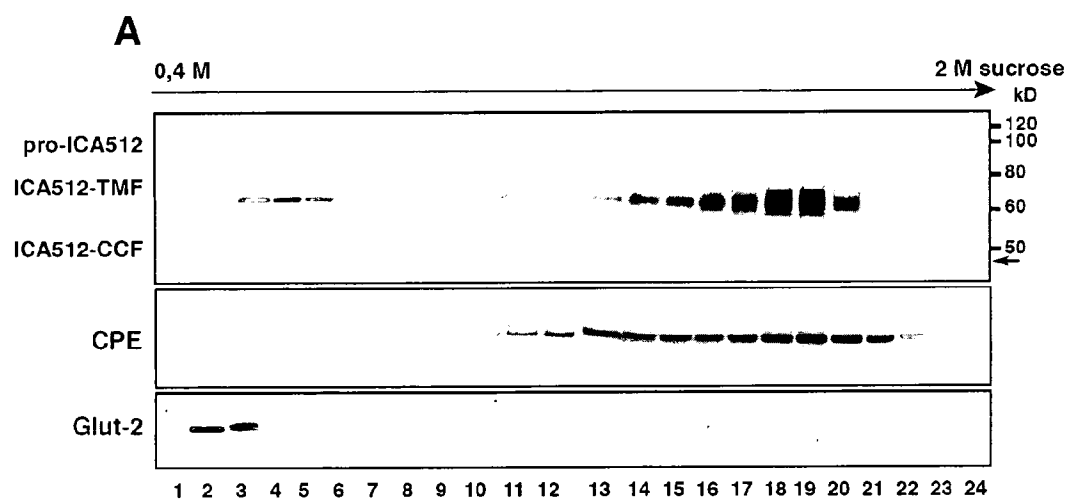
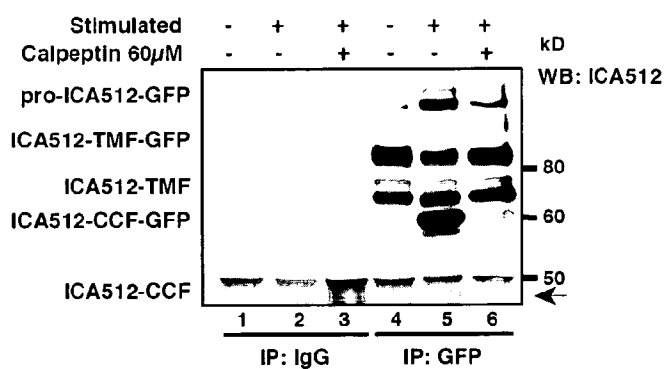
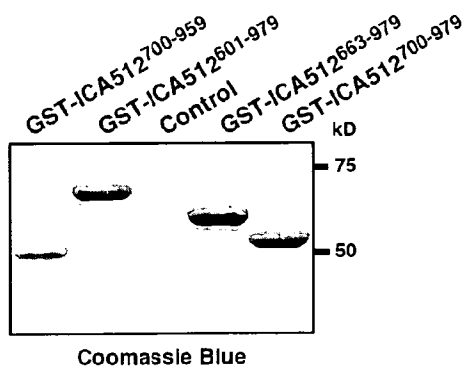
**Figure 7**

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**Figure 8**

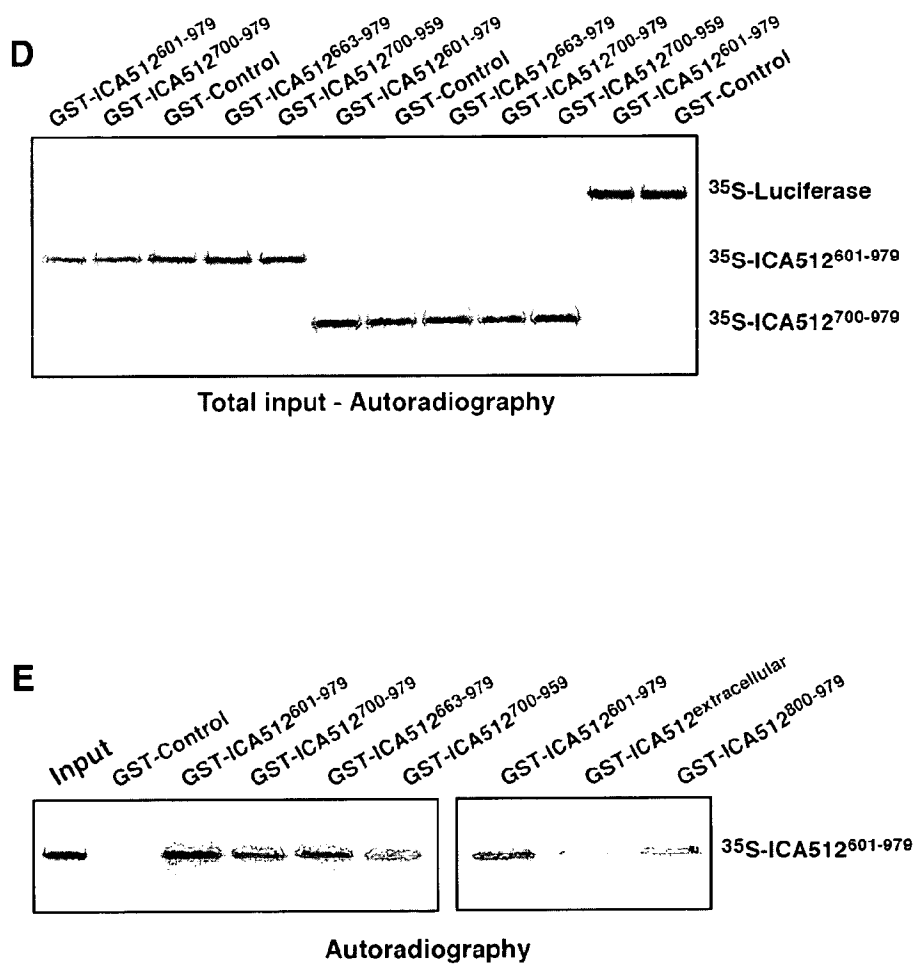
**Figure 8**

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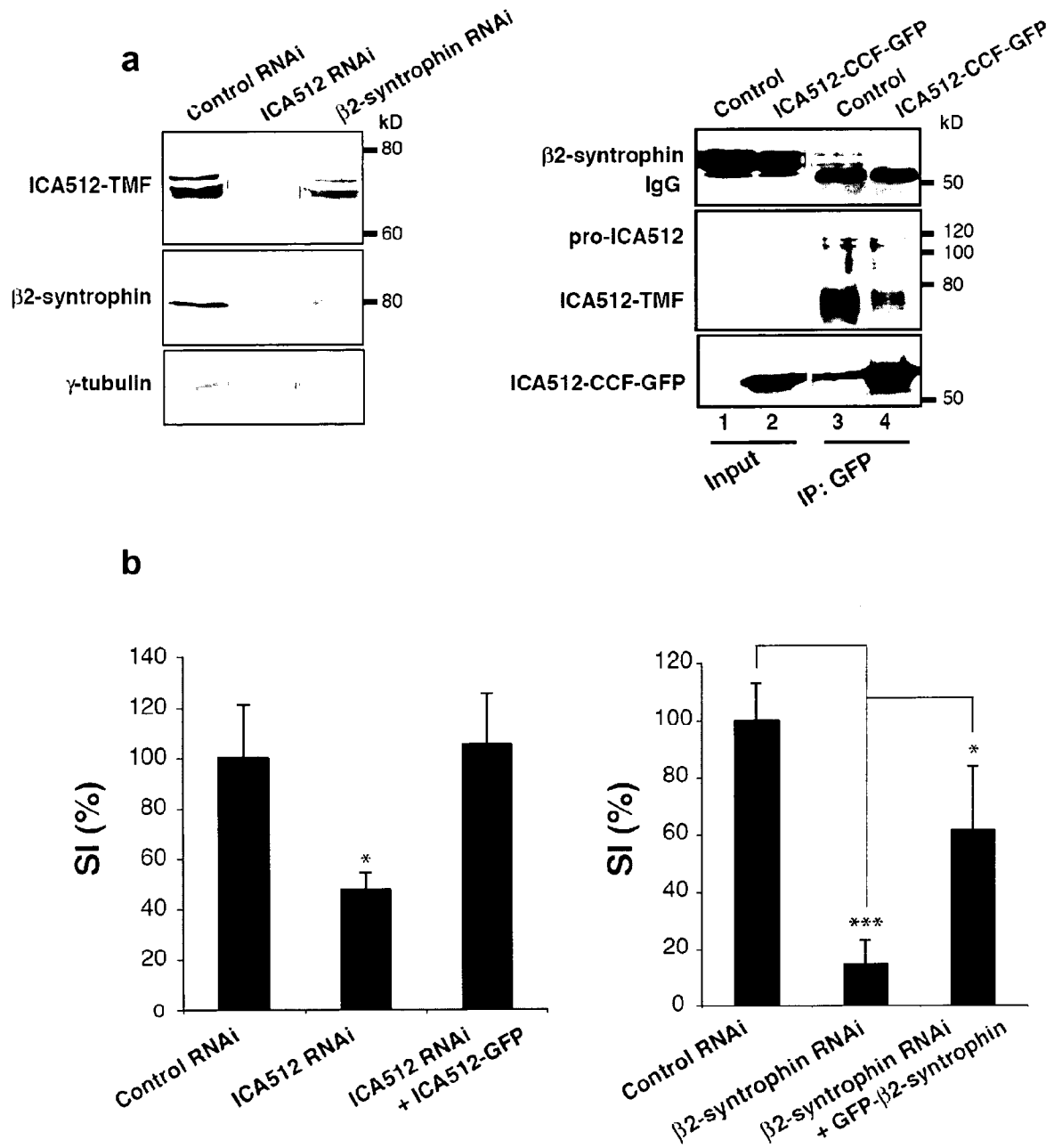
**Figure 9****B****C**

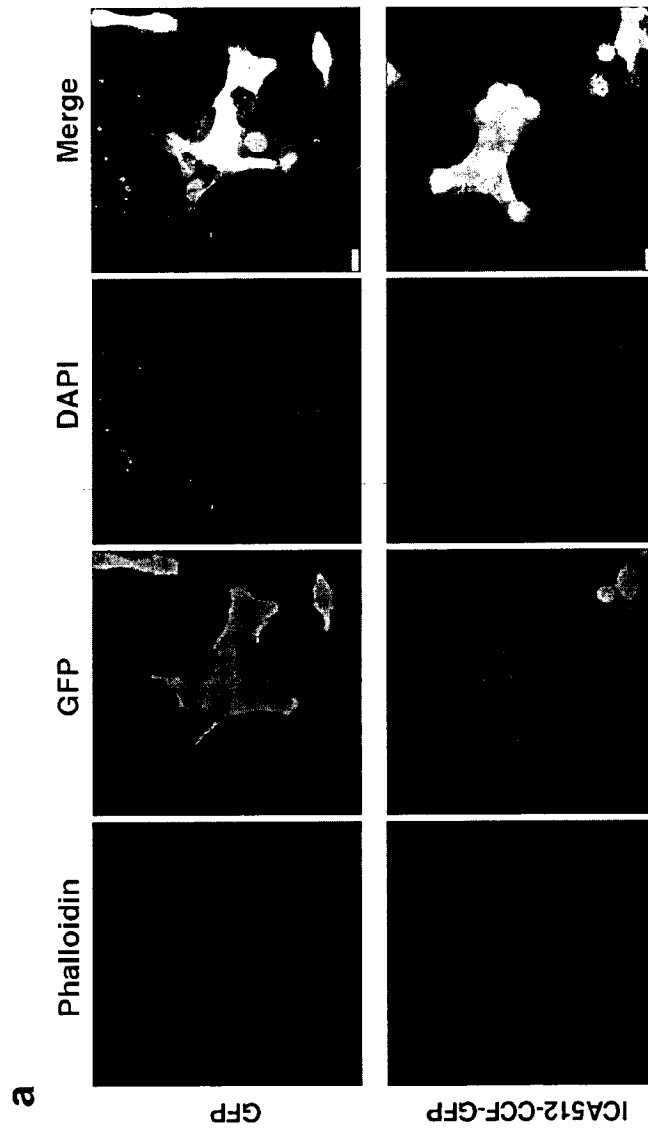
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**Figure 9**



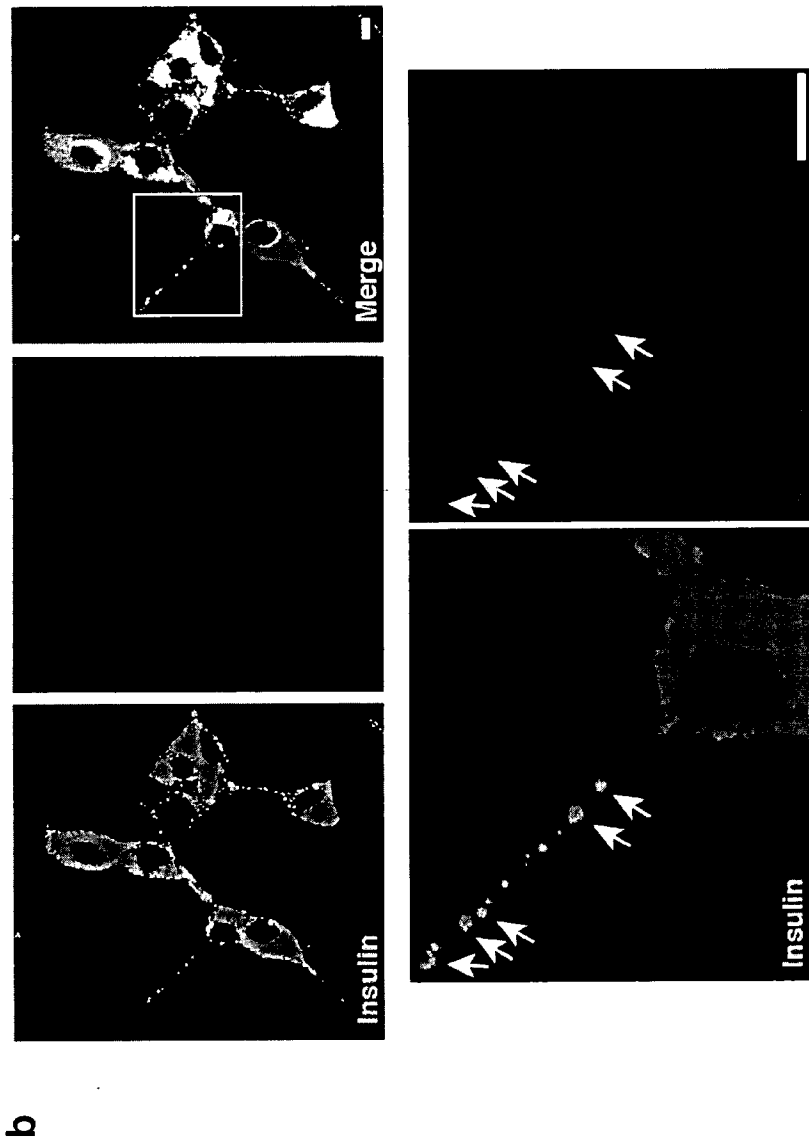
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**Figure 10**



**Figure 11**

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**Figure 11**

# INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/003557

## A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 G01N33/50 A61K31/66 A61P3/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                   | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

16 September 2008

Date of mailing of the international search report

24/09/2008

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| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                               | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
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# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2008/003557

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                | Relevant to claim No. |
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Information on patent family members

International application No

PCT/EP2008/003557

| Patent document<br>cited in search report |    | Publication<br>date | Patent family<br>member(s)                                                                                                               | Publication<br>date                                                                                          |
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