

Nuclear translocation of an ICA512 cytosolic fragment couples granule exocytosis and insulin expression in β -cells

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Islet cell autoantigen 512 (ICA512)/IA-2 is a receptor tyrosine phosphatase-like protein associated with the insulin secretory granules (SGs) of pancreatic β -cells. Here, we show that exocytosis of SGs and insertion of ICA512 in the plasma membrane promotes the Ca^{2+} -dependent cleavage of ICA512 cytoplasmic domain by μ -calpain. This cleavage occurs at the plasma membrane and generates an ICA512 cytosolic fragment that is

targeted to the nucleus, where it binds the E3-SUMO ligase protein inhibitor of activated signal transducer and activator of transcription- γ (PIAS γ) and up-regulates insulin expression. Accordingly, this novel pathway directly links regulated exocytosis of SGs and control of gene expression in β -cells, whose impaired insulin production and secretion causes diabetes.

Introduction

Neuroendocrine cells, including endocrine cells of the pancreatic islets, modulate physiological activities through the regulated secretion of peptide hormones and neuropeptides, such as insulin. These signaling molecules are stored intracellularly within secretory granules (SGs) that fuse with the plasma membrane in response to various stimuli and release extracellularly their content. Insulin-containing SGs of pancreatic β -cells, in particular, undergo exocytosis when blood glucose levels are ≥ 5.5 mM. As insulin lowers blood glucose levels, deficits of its production, secretion, or signaling cause hyperglycemia and diabetes, the most common metabolic disorder in humans. Still unclear is how pancreatic β -cells, and neuroendocrine cells in general, constantly monitor their own secretory activity and adjust their hormone and gene expression profile accordingly.

Islet cell autoantigen 512 (ICA512; also known as IA-2) is an intrinsic membrane protein of insulin SGs and neurosecretory granules in general (Solimena et al., 1996). This type

I transmembrane protein, which is a major autoimmune target in type 1 diabetes (Lan et al., 1994; Rabin et al., 1994), belongs to the receptor protein tyrosine phosphatase (PTP) family due to the inclusion of a single PTP cytosolic domain lacking phosphatase activity (Magistrelli et al., 1996). Deletion of ICA512 in mice impairs insulin secretion (Saeki et al., 2002) for reasons that are unclear. In view of its relationship with receptor tyrosine phosphatases and SGs, we investigated here whether or not ICA512 links regulated secretion and signal transduction in β -cells. Our findings indicate that the exocytosis of insulin SGs triggers the cleavage of ICA512 cytoplasmic domain, which, in turn, translocates to the nucleus and promotes insulin gene expression.

Results

Cleavage of ICA512 upon stimulation of insulin secretion

Pro-ICA512 is a glycoprotein of 110 kD. Processing of its ectodomain by a furin-like protease during SG maturation converts pro-ICA512 into the mature ICA512-transmembrane fragment (ICA512-TMF) of 65 kD, which is found on SGs (Solimena et al., 1996). Stimulation of rat insulinoma cells-1 (INS-1) with high glucose (25 mM) and high K^+ (55 mM) induces pro-ICA512 biosynthesis and reduces ICA512-TMF levels (Ort et al., 2001; Fig. 1 A, lanes 1 and 2). This dual effect

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Abbreviations used in this paper: APP, amyloid precursor protein; CPE, carboxypeptidase E; FRET, fluorescence resonance energy transfer; ICA512, Islet Cell Autoantigen 512; ICA512-CCF, ICA512-cleaved cytosolic fragment; ICA512-TMF, ICA512-transmembrane fragment; INS-1, insulinoma cells-1; PIAS, protein inhibitor of activated STAT; PTP, protein tyrosine phosphatase; SG, secretory granule; siRNA, short-interfering RNA; STAT, signal transducer and activator of transcription.

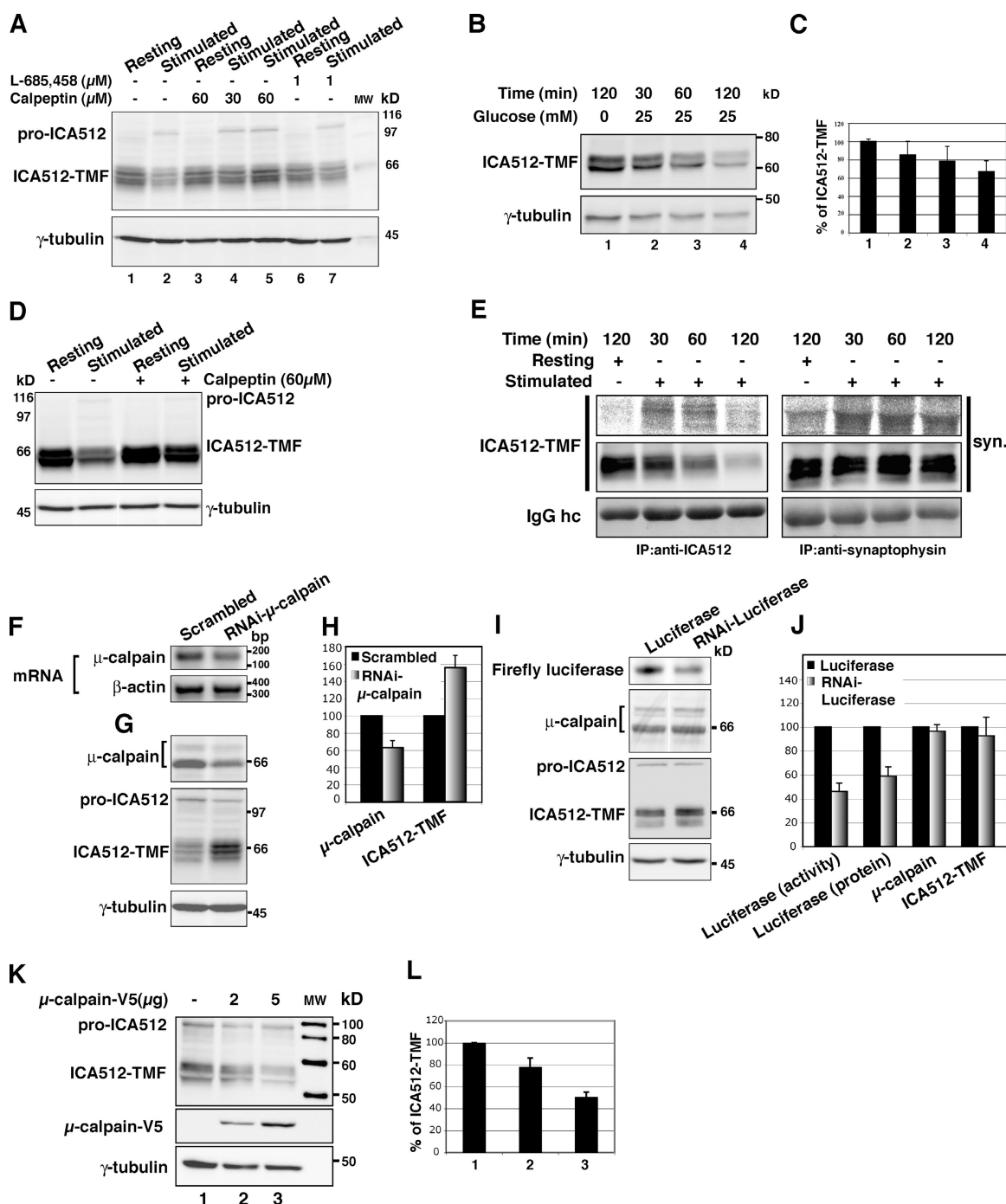


Figure 1. ICA512 cytoplasmic domain is cleaved by μ -calpain in response to stimulation of β -cells. (A) Western blot for ICA512 (top) and γ -tubulin (bottom) on 40 μ g of protein from INS-1 cells that had been incubated with resting or stimulating buffer for 105 min following 1 h at rest. Protease inhibitors L-685,458 and calpeptin were added at indicated concentrations. (B) Western blots for ICA512 (top) and γ -tubulin (bottom) on 30 μ g of protein from rat islets that were incubated with 0 (resting) or 25 mM (stimulated) glucose for indicated times. (C) Quantitation of ICA512-TMF from two independent experiments as shown in B. (D) Western blots for ICA512 (top) and γ -tubulin (bottom) on 30 μ g of protein from rat islets that were incubated in resting or stimulating buffers as in A, with or without 60 μ M calpeptin. White lines indicate that intervening lanes have been spliced out. (E) Autoradiographies showing the turnover of newly synthesized ICA512-TMF (top left) and synaptophysin (syn.; top right) as determined by pulse-chase and immunoprecipitation from [35 S]methionine-labeled INS-1 cells kept at rest or stimulated for the indicated times. Middle and bottom panels show the total amount of ICA512-TMF (left middle), synaptophysin (right middle), and IgG heavy chain (bottom panels) in each immunoprecipitate as visualized by Western blotting. (F) Levels of μ -calpain and β -actin mRNAs by semiquantitative RT-PCR in INS-1 cells transfected with scrambled or anti- μ -calpain siRNA oligos 1. (G) Western blots for μ -calpain (top), pro-ICA512 (middle), ICA512-TMF (bottom), and γ -tubulin (bottom) on 40 μ g of protein from stimulated INS-1 cells transfected with scrambled or anti- μ -calpain siRNA oligos 1. (H) Quantitation of μ -calpain and ICA512-TMF from three independent experiments as shown in G. (I) Western blots for firefly luciferase (top), μ -calpain (middle top), pro-ICA512 (middle bottom), ICA512-TMF (bottom), and γ -tubulin (bottom) on 30 μ g of protein in INS-1 cells transfected with firefly luciferase cDNA and untreated or treated with siRNA oligos for firefly luciferase. White lines indicate that intervening lanes have been spliced out. (J) Firefly luciferase activity and protein levels of firefly luciferase, μ -calpain, and ICA512-TMF from three independent experiments as shown in I. (K) Western blots for ICA512 (top), μ -calpain-V5 (middle), and γ -tubulin (bottom) on 40 μ g of protein from INS-1 cells transfected with the indicated amount of cDNA for μ -calpain-V5 and stimulated as in A. (L) Quantitation of ICA512-TMF from three independent experiments as shown in K. In B, D, H, J, and L, protein signals were normalized for γ -tubulin and expressed as a percentage of their respective values in cells at rest (B and D), transfected with scrambled siRNA oligos (H) or firefly luciferase cDNA (J), or electroporated (L). Error bars in C, H, J, and L show mean $\pm$ SD.

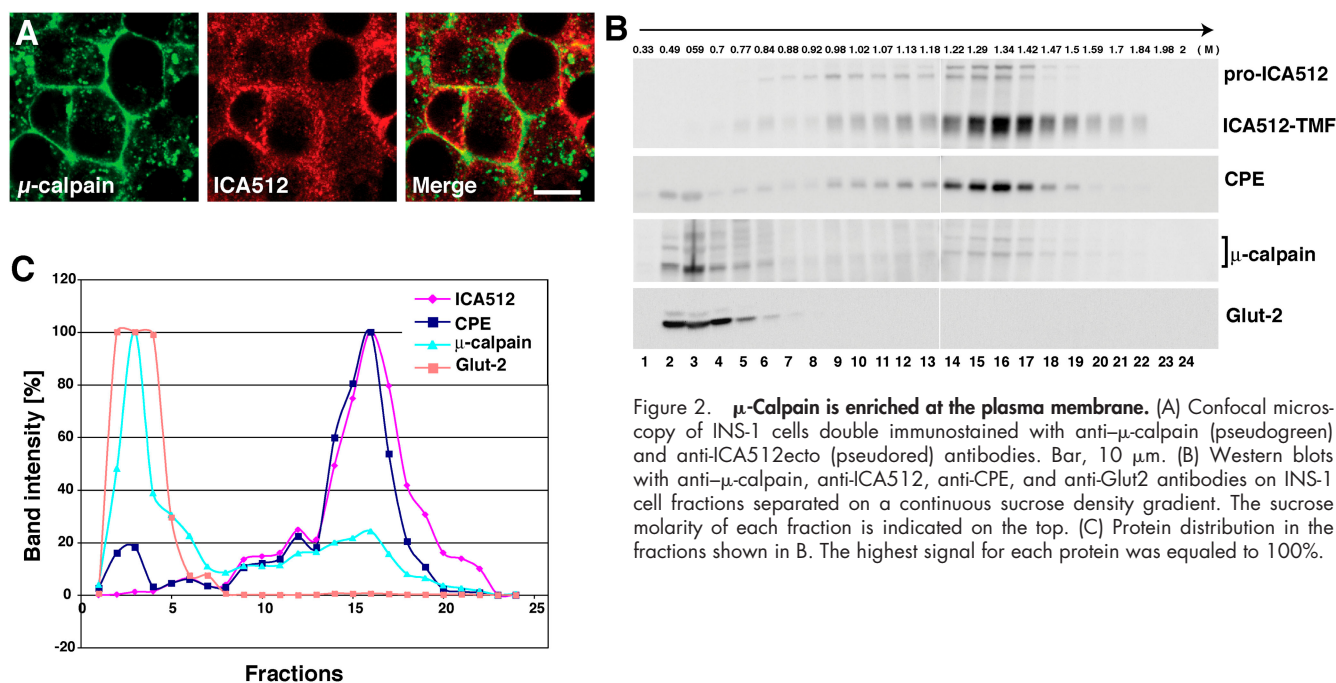


Figure 2. μ -Calpain is enriched at the plasma membrane. (A) Confocal microscopy of INS-1 cells double immunostained with anti- μ -calpain (pseudogreen) and anti-ICA512ecto (pseudoredd) antibodies. Bar, 10 μ m. (B) Western blots with anti- μ -calpain, anti-ICA512, anti-CPE, and anti-Glut2 antibodies on INS-1 cell fractions separated on a continuous sucrose density gradient. The sucrose molarity of each fraction is indicated on the top. (C) Protein distribution in the fractions shown in B. The highest signal for each protein was equaled to 100%.

on ICA512 can be dissociated by stimulating INS-1 cells either with high glucose or high K^+ (Ort et al., 2001). High glucose alone prompts pro-ICA512 biosynthesis, but only a modest release of insulin and decrease of ICA512-TMF. In contrast, high K^+ induces a strong insulin secretion and decrease of ICA512-TMF, but it does not enhance pro-ICA512 biosynthesis. The reduction of ICA512-TMF depends on the potency of a stimulus in inducing SG exocytosis. Stimulation with high glucose alone for 30, 60, and 120 min elicits indeed a progressive reduction of ICA512-TMF in freshly isolated rat pancreatic islets (Fig. 1, B and C), which, unlike INS-1 cells, retain a virtually intact secretory responsiveness to glucose. Pro-ICA512 induction by glucose is less apparent in islets (Fig. 1 D) than in INS-1 cells because they convert faster pro-ICA512 into ICA512-TMF. This induction, indeed, is readily detected upon stimulation of islets at 19°C, which blocks protein export from the trans-Golgi network and thereby pro-ICA512 conversion into ICA512-TMF (Knoch et al., 2004). Pulse-chase [35 S]methionine labelings in INS-1 cells show that newly synthesized ICA512-TMF has a faster turnover compared with synaptophysin, an intrinsic membrane protein of synaptic vesicle/synaptic-like microvesicles, which is known to participate in multiple rounds of exo-endocytosis (Johnston et al., 1989; Fig. 1 E). Notably, newly synthesized ICA512-TMF is virtually not detectable in INS-1 cells kept at rest for 2 h after 1 h labeling in medium with low glucose (5.5 mM), whereas it is readily apparent in cells only exposed for 30 min to high glucose (25 mM), which is consistent with the notion that glucose rapidly up-regulates ICA512 biosynthesis (Knoch et al., 2004).

Exocytosis of SGs leads to the insertion of ICA512-TMF in the plasma membrane (Solimena et al., 1996) and correlates with the decrease of ICA512-TMF because of the Ca^{2+} -dependent cleavage of its cytoplasmic domain by calpain (Ort et al., 2001). This cleavage is indeed prevented in a dose-dependent manner by calpeptin (Fig. 1 A, lanes 3–5), which inhibits

calpains (Tsujinaka et al., 1988), but not by inhibiting γ -secretase with L-685,458 (Shearman et al., 2000; Fig. 1 A, lanes 6–7) or lysosomal and proteasome activities (Ort et al., 2001). ICA512-TMF is also decreased in a calpeptin-sensitive fashion in stimulated islets (Fig. 1 D).

ICA512-TMF is cleaved by μ -calpain

Among the 14 known calpains, only μ -calpain is activated by micromolar concentrations of intracellular Ca^{2+} (Glading et al., 2002), i.e., within levels elicited by segretagogues to trigger SG exocytosis. INS-1 cells express μ -calpain, and calpain activity is enhanced by stimulation of insulin secretion (Ort et al., 2001). In vitro μ -calpain cleaves recombinant ICA512 cytoplasmic domain between residues 658–659 (Ort et al., 2001). This site follows a PEST motif (residues 643–651)—a common proteolytic mark among calpain substrates (Tomp et al., 2004). Both calpeptin and MG-132, another inhibitor of cysteine proteases, prevent this cleavage in vitro (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>). To establish whether or not μ -calpain is responsible for cleaving ICA512-TMF in stimulated cells, its cytoplasmic mRNA (Fig. 1 F) and protein levels (Fig. 1, G and H) in INS-1 cells were knocked down using three distinct double-stranded short-interfering RNA (siRNA) oligonucleotides, either individually (Fig. 1, G and H) or in combination (Fig. S1 B). Decrease of μ -calpain inhibits the stimulus-dependent degradation of ICA512-TMF, whereas knock-down of transiently transfected firefly luciferase, as verified by immunoblot and enzymatic assay (Fig. 1, I and J), does not affect μ -calpain or ICA512-TMF expression. Conversely, overexpression of μ -calpain increases ICA512-TMF proteolysis only upon stimulation (Fig. 1, K and L; and Fig. S1 C). These data prove that μ -calpain cleaves ICA512-TMF in stimulated cells.

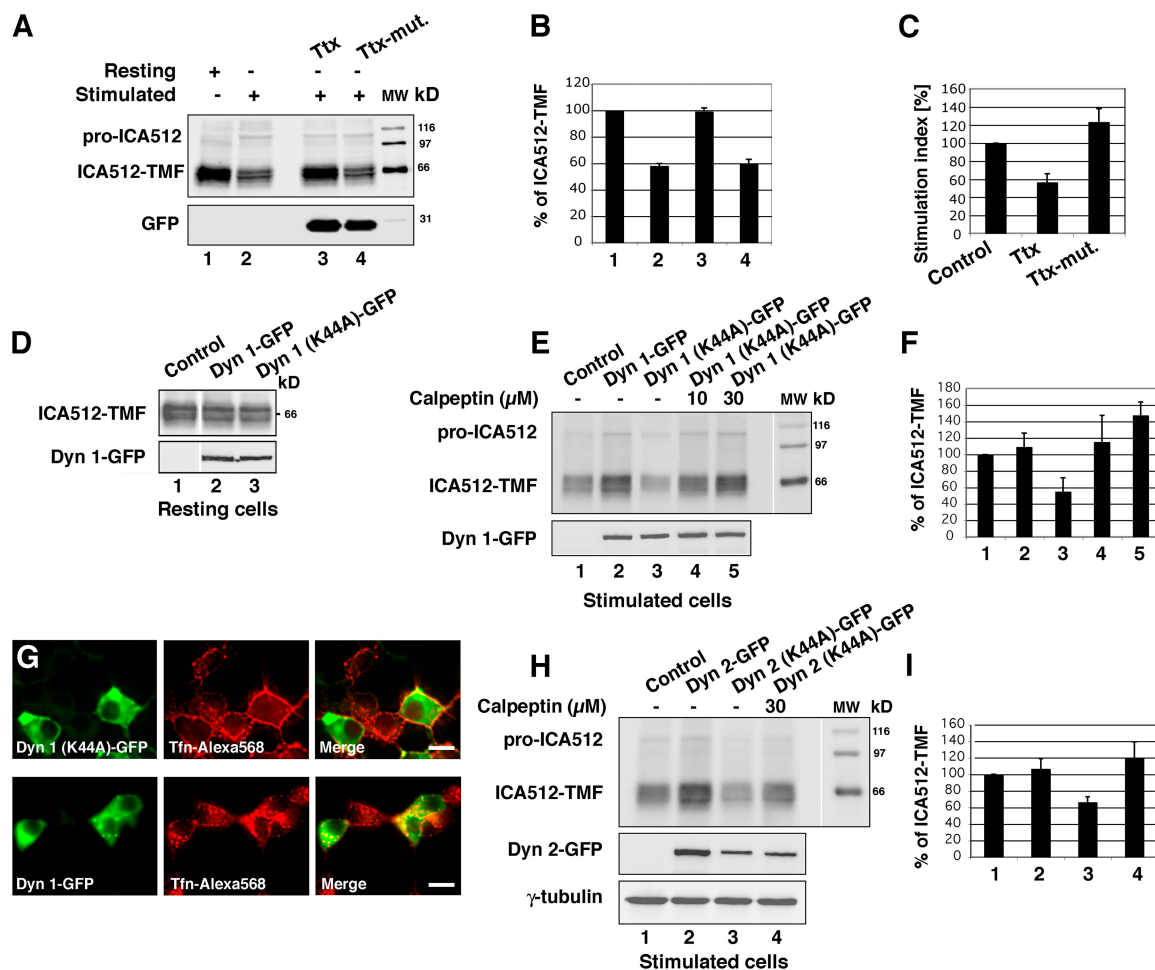


Figure 3. ICA512 is cleaved by μ -calpain at the plasma membrane. (A) Western blottings with anti-ICA512 (top) or GFP (bottom) antibodies on 40 μ g of protein from INS-1 cells cotransfected with active (Ttx) or inactive (Ttx-mut.) tetanus toxin light chain and GFP. Cells were incubated in resting or stimulating buffer for 105 min after 1 h at rest. (B) Quantitation of ICA512-TMF from three independent experiments as shown in A. (C) Stimulation index of insulin secretion from INS-1 cells that were either nontransfected or transfected with Ttx or Ttx-mut. (D) Western blotting with anti-ICA512 (top) and anti-GFP (bottom) antibodies on 30 μ g of protein from INS-1 cells, which were either nontransfected (control) or transiently transfected with dynamin 1-GFP or dynamin 1 (K44A)-GFP and kept in resting buffer for 1 h. White lines indicate that intervening lanes have been spliced out. (E and H) Western blotting with anti-ICA512 (top) and anti-GFP (bottom) antibodies on 20 μ g of protein from INS-1 cells, which were either nontransfected (control) or transiently transfected dynamin 1-GFP or dynamin 1 (K44A)-GFP (E) and with dynamin 2-GFP or dynamin 1 (K44A)-GFP (H). Cells were stimulated for 105 min in the absence or presence of calpeptin at the indicated concentration. As the expression of dynamin 2 constructs differed, loading of comparable protein amount was verified by immunoblot for γ -tubulin. (F and I) Quantitation of ICA512-TMF from four independent experiments as shown in E and H, respectively. Error bars in B, C, F, and I show mean $\pm$ SD. (G) Fluorescence microscopy on INS-1 cells transiently transfected with dynamin 1 (K44A)-GFP (top) or dynamin 1-GFP (bottom) and incubated with Alexa⁵⁶⁸-transferrin (TfR-Alexa568). Bars, 10 μ m.

ICA512-TMF is cleaved at the plasma membrane

μ -Calpain is recruited to the plasma membrane and activated by Ca^{2+} and phospholipids, which are both implicated in neurosecretion (Martin, 1998; Wenk and De Camilli, 2004). In INS-1 cells, μ -calpain is mostly restricted to the cortical region (Fig. 2 A, left), where SGs are also enriched, as shown by staining for ICA512 (Fig. 2 A, middle). After subcellular fractionation, most μ -calpain is recovered in fractions containing the plasma membrane marker Glut-2 (Fig. 2, B and C), although a minor pool is found in the same fractions with ICA512-TMF and carboxypeptidase E (CPE), another marker of SGs.

To directly address whether ICA512-TMF cleavage precedes or follows SG exocytosis, INS-1 cells were transiently transfected with the light chain of clostridium tetanus toxin,

which blocks the last step in SG fusion with the plasma membrane by cleaving synaptobrevin 2/VAMP2 (Schiavo et al., 1992; Niemann et al., 1994; Regazzi et al., 1995). The active, but not the inactive, tetanus toxin light chain, similarly inhibits ICA512-TMF cleavage (Fig. 3 A) and insulin secretion (Fig. 3 C), thereby indicating that SG exocytosis and ICA512-TMF insertion in the plasma membrane are necessary in order for the latter to become susceptible to cleavage.

To test if the cleavage of ICA512-TMF follows its internalization, INS-1 cells were transiently transfected with GFP-tagged dominant-negative mutant of dynamin 1 (K44A) or dynamin 2 (K44A), which block clathrin-mediated endocytosis (van der Bliek et al., 1993; Holroyd et al., 2002). Expression of dynamin 1-GFP or dynamin 1 (K44A)-GFP does not alter ICA512-TMF levels in resting INS-1 cells (Fig. 3 D). In stimulated cells, in-

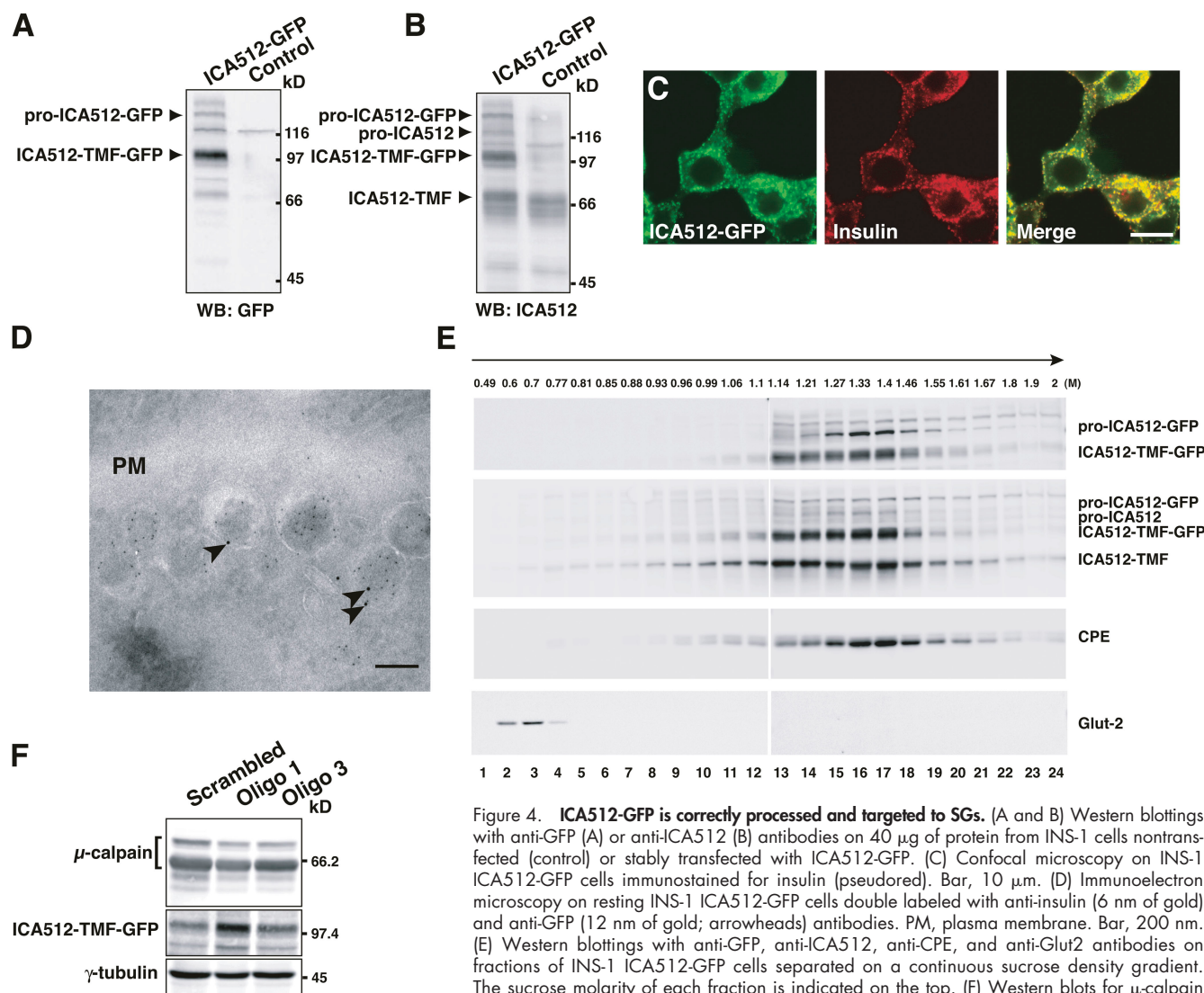


Figure 4. ICA512-GFP is correctly processed and targeted to SGs. (A and B) Western blottings with anti-GFP (A) or anti-ICA512 (B) antibodies on 40 μ g of protein from INS-1 cells nontransfected (control) or stably transfected with ICA512-GFP. (C) Confocal microscopy on INS-1 ICA512-GFP cells immunostained for insulin (pseudocolored). Bar, 10 μ m. (D) Immunoelectron microscopy on resting INS-1 ICA512-GFP cells double labeled with anti-insulin (6 nm of gold) and anti-GFP (12 nm of gold; arrowheads) antibodies. PM, plasma membrane. Bar, 200 nm. (E) Western blottings with anti-GFP, anti-ICA512, anti-CPE, and anti-Glut2 antibodies on fractions of INS-1 ICA512-GFP cells separated on a continuous sucrose density gradient. The sucrose molarity of each fraction is indicated on the top. (F) Western blots for μ -calpain (top), ICA512 (middle), and γ -tubulin (bottom) on 40 μ g of protein from INS-1 cells transfected with scrambled or anti- μ -calpain siRNA oligos 1 or 3.

stead, dynamin 1-GFP increases the stability of ICA512-TMF (Fig. 3 E), whereas dynamin 1 (K44A)-GFP decreases it, and this reduction is inhibited by calpeptin in a dose-dependent fashion (Fig. 3 E). Similar results are observed upon expression of wild-type or dominant-negative dynamin 2-GFP (Fig. 3, H and I). Uptake experiments show the internalization of Alexa⁵⁶⁸-labeled transferrin in INS-1 cells either nontransfected or transfected with dynamin 1-GFP or dynamin 2-GFP (Fig. 3 G and Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>), but not in cells expressing dynamin 1- or dynamin 2 (K44A)-GFP (Fig. 3 G and Fig. S2). Together, these results demonstrate that ICA512-TMF is cleaved at the plasma membrane, where μ -calpain is enriched (Fig. 2 B) and activated by Ca^{2+} , whereas endocytosis spares ICA512-TMF from cleavage.

ICA512-GFP is correctly sorted and processed

Recognition by the anti-ICA512 monoclonal antibody of a juxtamembrane epitope just upstream of the calpain cleavage site

(Ort et al., 2001) precluded its use for investigating the fate of the ICA512-cleaved cytosolic fragment (ICA512-CCF). We overcame this limit by fusing GFP at the COOH terminus of ICA512. Immunoblots with anti-GFP or anti-ICA512 antibodies on extracts from stable ICA512-GFP INS-1 cells show pro-ICA512-GFP and ICA512-TMF-GFP having the expected sizes of 135 and 90 kD, respectively (Fig. 4, A and B). By confocal microscopy it appears that most ICA512-GFP colocalizes with insulin (Fig. 4 C). Specific labeling of granules with GFP antibody is also found by immunogold electron microscopy on cryosections (Fig. 4 D). In ICA512-GFP INS-1 cells, 19/87 granules were labeled, compared with 0/102 in nontransfected INS-1 cells ($P < 0.001$). Enrichment of ICA512-GFP on SGs has also been confirmed by subcellular fractionation on sucrose density gradients (Fig. 4 E). Moreover, limited knockdown of μ -calpain is sufficient to decrease ICA512-TMF-GFP cleavage (Fig. 4 F and Fig. S1 D). Thus, the GFP tag does neither prevent the conversion of pro-ICA512 into ICA512-TMF nor the targeting of the latter to SGs and its cleavage by μ -calpain.

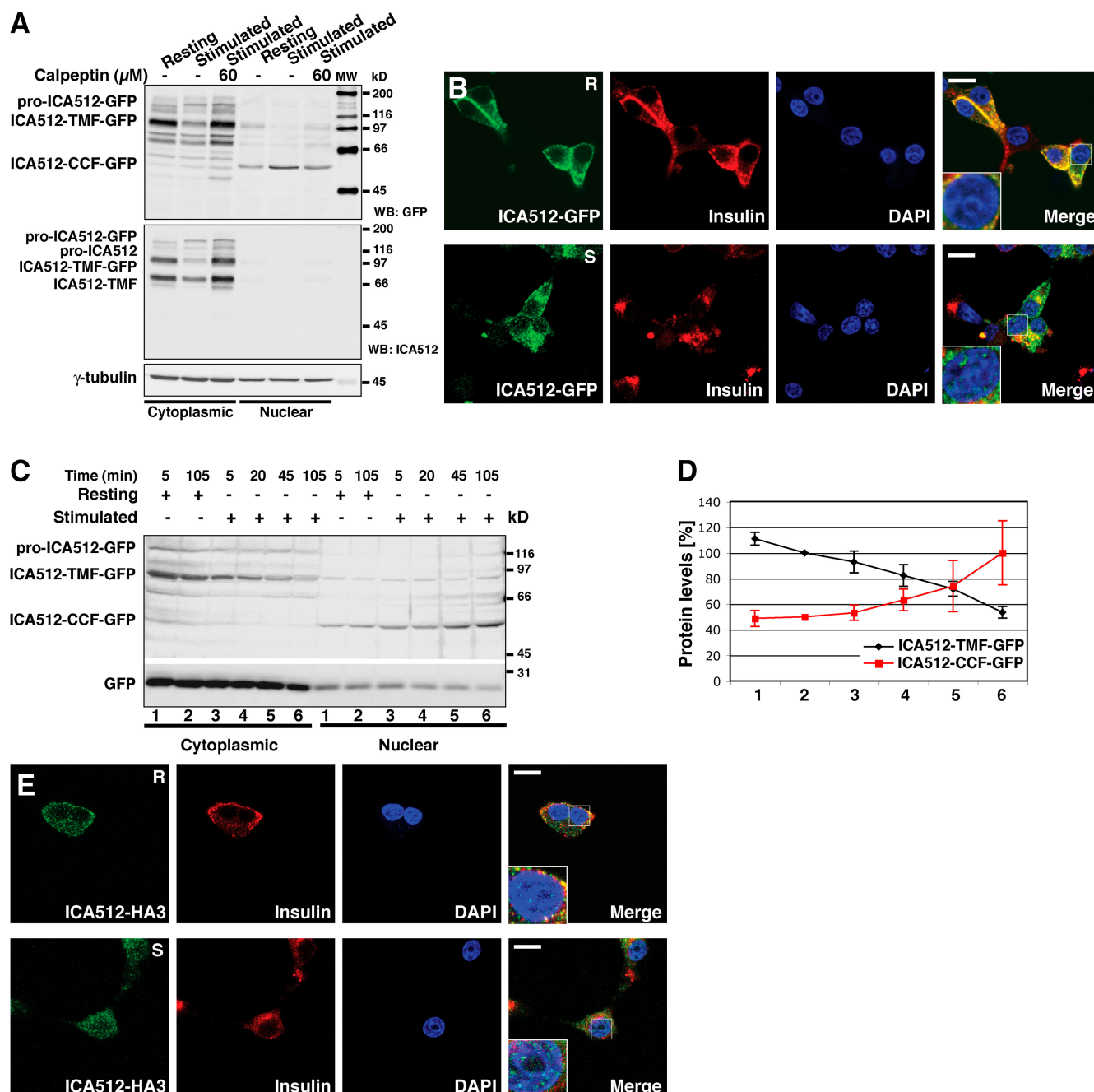


Figure 5. ICA512-CCF is translocated to the nucleus upon stimulation. (A) Western blots with anti-GFP (top), anti-ICA512 (middle), and γ -tubulin (bottom) on 50 μ g of cytoplasmic and 25 μ g of nuclear protein from INS-1 ICA512-GFP cells. Cells were incubated with resting or stimulating buffer for 105 min after 1 h at rest and with or without 60 μ M calpeptin. (B) 0.5- μ m optical Z-sections of INS-1 ICA512-GFP cells kept at rest (R; top) or stimulated (S; bottom) as in A and immunolabeled with anti-insulin antibody (pseudoredd). ICA512-GFP is shown as pseudogreen, whereas nuclei were counterstained with DAPI (pseudoblue). Insets in the right panels show high magnifications of the marked areas. (C) Western blots with anti-GFP on 40 μ g of cytoplasmic and 20 μ g of nuclear protein from INS-1 ICA512-GFP cells incubated as in A for the indicated times. (D) Quantitation of ICA512-TMF-GFP and ICA512-CCF-GFP in cytoplasmic (black line) and nuclear (red line) extracts at the indicated time points 1–6. Values are from four independent experiments as shown in C. For graphic representation, the amounts of ICA512-TMF-GFP and ICA512-CCF-GFP after 105 min in resting buffer (time point 2) were equalized to 100% and 50%, respectively. Error bars show mean $\pm$ SD. (E) 0.5- μ m optical Z-sections of INS-1 cells transiently transfected with ICA512-HA, kept at rest (R; top) or stimulated (S; bottom) as in A, and immunolabeled with rabbit anti-HA (pseudogreen) and mouse anti-insulin (pseudoredd) antibodies. Nuclei were counterstained with DAPI (pseudoblue). Insets in the right panels show high magnifications of the marked areas. Bars, 10 μ m.

ICA512-CCF is targeted to the nucleus

The stimulation-dependent proteolysis of ICA512-TMF-GFP is calpeptin-sensitive (Fig. 5 A, top panel). Strikingly, a GFP-reacting protein, whose size of 55 kD matches the one expected for ICA512-CCF-GFP, is enriched in nuclear, but not in cytosolic,

fractions of stimulated ICA512-GFP INS-1 cells, also in a calpeptin-sensitive fashion (Fig. 5 A, top panel, lanes 4–6). Predictably, this 55-kD protein is not recognized by the anti-ICA512 monoclonal antibody (Fig. 5 A, middle panel). ICA512-CCF-GFP is already present in the nuclei of cells at rest for 165 min.

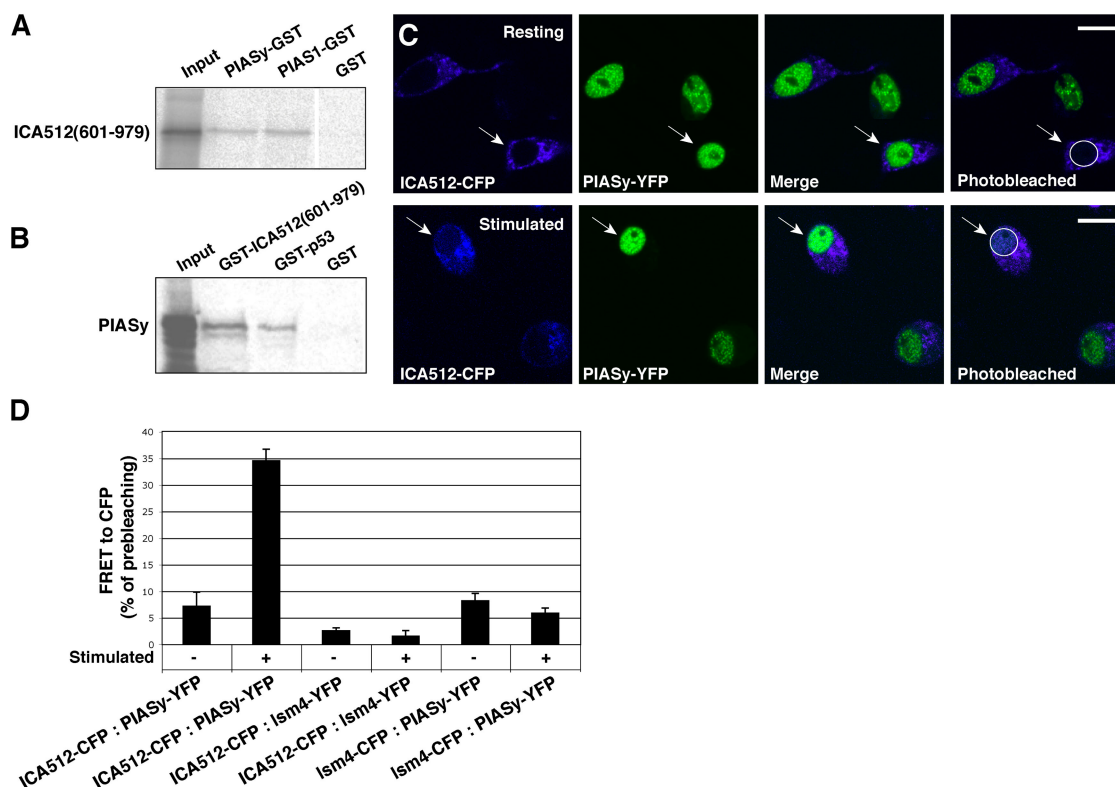


Figure 6. ICA512-CCF binds PIASy in the nucleus. (A) SDS-PAGE and autoradiography of in vitro transcribed and translated [35 S]methionine-labeled ICA512(601–979) pulled down with glutathione sepharose beads coupled to 0.5 μ g PIASy-GST, PIAS1-GST, or GST alone. Lane 1 shows the starting labeled material for the pull-down assay. White lines indicate that intervening lanes have been spliced out. (B) SDS-PAGE and autoradiography of in vitro transcribed and translated [35 S]methionine-labeled PIASy pulled down with glutathione sepharose beads coupled to 0.5 μ g GST-ICA512(601–979), GST-p53, or GST alone. First lane shows the starting labeled material for the pull-down assay. (C) FRET between ICA512-CFP (pseudocyan) and PIASy-YFP (pseudogreen) in transiently cotransfected INS-1 cells incubated in resting or stimulating buffer for 105 min after 1 h at rest. The right panels show the signal for ICA512-CFP following the photobleaching of PIASy-YFP in the circled nuclei (arrows). (D) Quantitation of FRET to CFP in INS-1 cells transiently cotransfected with the indicated constructs: ICA512-CFP + PIASy-YFP; ICA512-CFP + lsm4-YFP; lsm4-CFP + PIASy-YFP. Values are from two independent experiments as shown in C. (D) Error bars show mean + SD.

Conceivably, this time is insufficient to clear the nuclei from ICA512-CCF-GFP that is constantly generated in cells cultured with 11 mM glucose, which stimulates sub-maximal exocytosis.

Confocal microscopy shows an accumulation of GFP-positive dots in the nuclei of stimulated INS-1 ICA512-GFP cells versus those of resting cells (Fig. 5 B, insets; and Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>). Concomitantly, the cytosolic colocalization of ICA512-GFP and insulin is decreased, reflecting the reduced insulin content and the internalization of ICA512-TMF-GFP in stimulated cells. The accumulation of ICA512-CCF-GFP in the nuclei of stimulated cells correlates with the increased reduction of ICA512-TMF-GFP over time (Fig. 5, C and D) and is already detectable after 20 min stimulation (Fig. 5 D).

ICA512-CCF does not contain a canonic nuclear import signal. To rule out the possibility that its nuclear translocation results from its tagging with GFP, we analyzed the distribution of GFP alone as well as that of an ICA512 construct carrying as a distinct tag a triple HA epitope at its COOH terminus (ICA512-HA3). Nuclear extracts of stimulated cells contain less GFP than those of resting cells (Fig. 5 C and Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>). ICA512-HA3, which is correctly processed (Fig. S4 A, available

at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>), is also enriched in the nuclei of stimulated cells, as shown by immunoblotting (Fig. S4 B) and confocal microscopy with an anti-HA antibody (Fig. 5 E and Videos 3 and 4). Together, these data indicate that SG exocytosis and ICA512-TMF cleavage at the plasma membrane are coupled to the generation of ICA512-CCF, which is targeted to the nucleus.

ICA512-CCF binds protein inhibitor of activated STAT (PIAS)-y in the nucleus

Screening by 2-hybrid assays in yeast showed that the cytoplasmic domain of ICA512 binds PIASy (unpublished data). PIASs were originally identified as PIAS (Liu et al., 1998) and have been more recently shown to have E3-sumoylating activity toward numerous transcription factors, including p53 and signal transducers and activators of transcription (STATs; Johnson and Gupta, 2001; Takahashi et al., 2001; Melchior and Hengst, 2002; Schmidt and Muller, 2002). The binding of ICA512 cytoplasmic domain to PIAS proteins, including PIASy and PIAS1, was confirmed by pull-down assays (Fig. 6 A). Specifically, 35 S-labeled human ICA512 cytoplasmic domain (residues 601–979) generated by in vitro transcription and translation is recovered on glutathione-sepharose beads coupled with PIASy-GST or PIAS1-

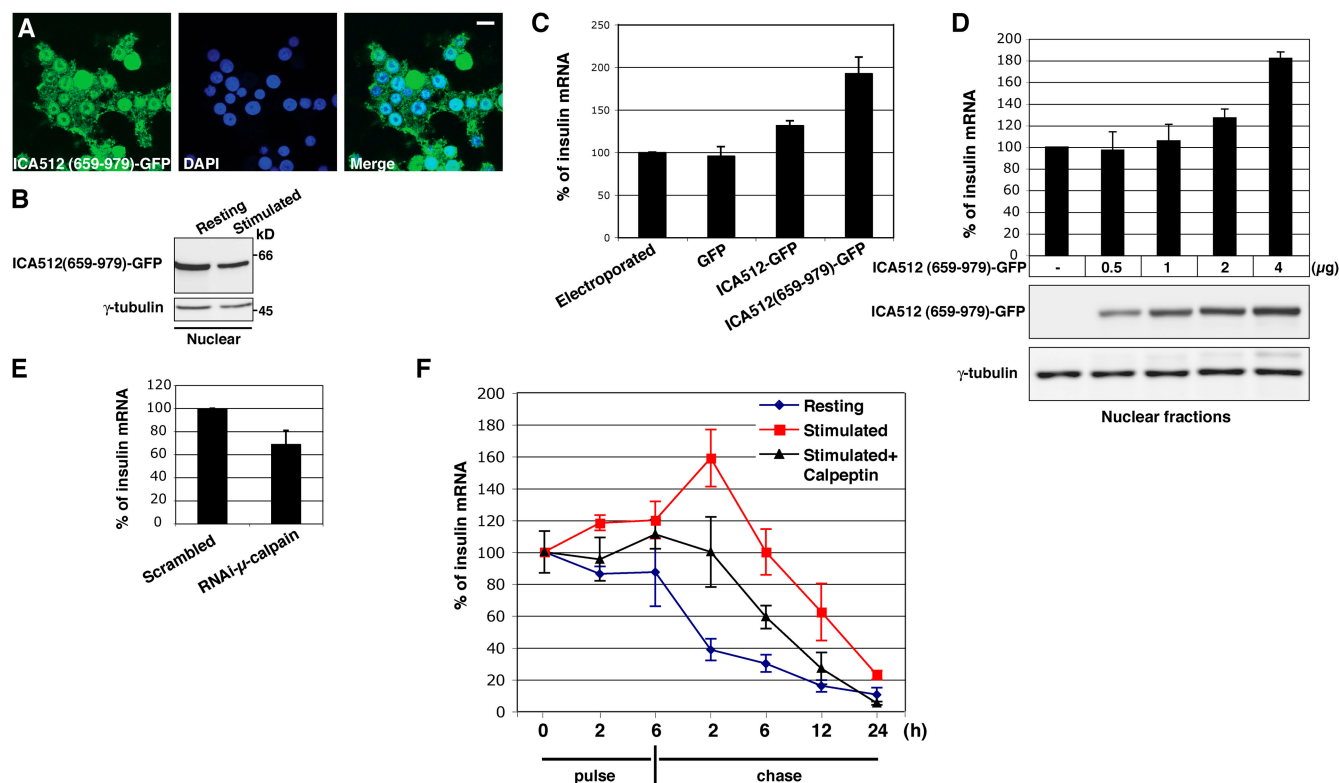


Figure 7. ICA512-CCF promotes insulin gene expression. (A) Confocal microscopy on INS-1 cells transiently transfected with ICA512 (659–979)-GFP and counterstained with DAPI. Bar, 10 μ m. (B) Western blot with goat anti-GFP (top) and anti- γ -tubulin (bottom) antibodies on 6 μ g of nuclear protein from INS-1 cells transiently transfected with ICA512(659–979)-GFP and incubated with resting or stimulating buffer for 105 min after 1 h at rest. (C) Insulin mRNA levels quantified by real-time PCR in INS-1 cells that were either electroporated only or transfected with 4 μ g of vector encoding GFP, ICA512-GFP, or ICA512(659–979)-GFP and kept at rest for 1 h. The results are from three independent experiments, each in triplicate. (D) Insulin mRNA levels quantified by real-time PCR in INS-1 cells which were either electroporated only or transfected with 0.5–4 μ g ICA512(659–979)-GFP and collected after 4 d in culture with 11 mM glucose. The results are from four independent experiments, each in triplicate. In C and D, the levels of insulin mRNA in electroporated cells were equaled to 100%. In D, ICA512(659–979)-GFP and γ -tubulin levels were assessed by Western blotting with goat anti-GFP (middle) and anti- γ -tubulin (bottom) antibodies. (E) Insulin mRNA levels quantified by real-time PCR in INS-1 cells transfected either with scrambled or anti- μ -calpain siRNA oligos 1 + 2. The results are from three independent experiments, each in triplicate. The level of insulin mRNA in cells transfected with the scrambled oligos was equaled to 100%. Error bars (C, D, and E) show mean $\pm$ SD. (F) Insulin mRNA levels quantified by real-time PCR in INS-1 cells that were at rest (2.8 mM glucose and 5 mM KCl) for 1 h, then in resting or stimulating buffer, with or without 40 μ M calpeptin, for up to 6 h (pulse), and finally in resting buffer for up to 24 h (chase). Total RNA was collected at 0-, 2-, and 6-h time points of the pulse and then at 2-, 6-, 12-, and 24-h time points of the chase. The results are from three independent experiments, each in triplicate. The level of insulin mRNA in resting INS-1 cells was equaled to 100%. Error bars show mean $\pm$ SD.

GST, but not with GST alone (Fig. 6 A). Likewise, 35 S-labeled PIASy interacts specifically with GST-ICA512(601–979) and GST-p53 (Fig. 6 B). Fluorescence resonance energy transfer (FRET) analyses in INS-1 cells transiently cotransfected with ICA512-CFP and PIASy-YFP show that the two proteins interact in the nucleus (Fig. 6 C). Specifically, the ICA512-CFP nuclear signal after photobleaching (Fig. 6 C, circled areas) is increased approximately fivefold upon stimulation (Fig. 6 D). In parallel experiments, no interaction is detected between ICA512-CCF-CFP and the nuclear protein lsm4-YFP (Fig. 6 D and Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>) and between lsm4-CFP and PIASy-YFP (Fig. 6 D) in either resting or stimulating conditions, thereby confirming the specific interaction of ICA512-CCF with PIASy in the nucleus.

ICA512-CCF enhances insulin gene expression

To test whether or not ICA512-CCF is a retrograde signal that couples regulated secretion and gene expression, possibly

through PIASy, INS-1 cells were transiently transfected with the ICA512 cytoplasmic fragment generated in vitro by μ -calpain (Ort et al., 2001; Fig. S1), which was tagged at its COOH terminus with GFP (ICA512(659–979)-GFP). Notably, ICA512(659–979) includes the catalytically inactive PTP domain. Confocal microscopy (Fig. 7 A) and Western blotting (Fig. 7 B) show that the nuclei of resting and stimulated cells contain comparable amounts of ICA512(659–979). Strikingly, insulin mRNA levels are increased by 32 and 93% in resting INS-1 cells expressing ICA512-GFP or ICA512(659–979)-GFP, respectively, but are not changed by the expression of GFP alone (Fig. 7 C). The higher levels of insulin mRNA in ICA512-GFP cells can be attributed to an increased generation of ICA512-CCF-GFP, as insulin mRNA is enhanced in parallel with the expression of ICA512(659–979)-GFP (Fig. 7 D). Accordingly, knockdown of μ -calpain decreases insulin mRNA (Fig. 7 E). Likewise, calpeptin has an adverse effect on insulin mRNA in stimulated cells (Fig. 7 F). In these experiments, cells were either kept at rest or stimulated for up to 6 h, with or

without 40 μ M calpeptin, and then returned to resting medium for up to 24 h. Maximal levels of insulin mRNAs are present 2 h after the 6-h stimulation period. However, in calpeptin-treated cells, insulin mRNA is reduced by $\sim$ 40% at this time point as well as at any later time point compared with cells stimulated in the absence of calpeptin. The magnitude of this decrease correlates well with the degree of calpeptin-mediated inhibition of ICA512-TMF cleavage (Fig. 1 B).

Discussion

In this work, we have identified a novel retrograde pathway that directly links regulated exocytosis and gene expression through the cleavage of a receptor tyrosine phosphatase-like protein associated with insulin SGs. Specifically, we show that stimulation promotes the cleavage of ICA512-TMF by μ -calpain. The resulting ICA512-CCF translocates to the nucleus, where it binds PIASy and promotes insulin gene expression.

Our data demonstrate that ICA512-TMF is cleaved while resident in the plasma membrane, following SGs exocytosis and before its internalization. Multiple factors contribute to the temporal and spatial restriction of this process. First, activation of μ -calpain requires concentrations of intracellular Ca^{2+} that are only transiently induced by stimulation of exocytosis and only in close proximity of the plasma membrane. Second, ICA512-TMF cytoplasmic tail binds β 2-syntrophin on SGs in the cell cortex (Ort et al., 2000). β 2-Syntrophin, in turn, is associated with the F-actin binding protein utrophin. Therefore, the ICA512-TMF/ β 2-syntrophin/utrophin complex may anchor SGs with the cortical actin cytoskeleton (Ort et al., 2001; Solimena and Gerdes, 2003). Notably, the PDZ domain of β 2-syntrophin binds ICA512-TMF between the PEST motif and the PTP domain (Ort et al., 2000) and this binding prevents the *in vitro* processing of ICA512 cytoplasmic domain by μ -calpain (Ort et al., 2001), conceivably by masking the cleavage site. Furthermore, stimulation of exocytosis appears to promote a Ca^{2+} -dependent dephosphorylation of β 2-syntrophin and thereby its dissociation from ICA512 (Ort et al., 2001; unpublished data). Together, these data point to Ca^{2+} as a signal that elicits the fusion of SGs, the insertion of ICA512-TMF in the plasma membrane, its dissociation from β 2-syntrophin, and its cleavage by activated μ -calpain, which is either bound to the plasma membrane or already found on SG membranes in proximity of ICA512-TMF. Binding of ligands to the ICA512 extracellular domain upon its exposure at the cell surface might further regulate the intracellular cleavage of ICA512-TMF.

Glucose is the most important factor regulating the expression of insulin, ICA512, and most other components of β -cell SGs. Such regulation is a complex process, which depends on transcriptional and posttranscriptional mechanisms as well as on glucose metabolism and SG exocytosis. Posttranscriptional mechanisms account for the increased biosynthesis of insulin and most other SG proteins within 2 h after stimulation (Itoh and Okamoto, 1980; Welsh et al., 1986). Specifically, glucose induces the rapid nucleocytoplasmic translocation of polypyrimidine-tract binding protein, which binds mRNAs of SG components (Knoch et al., 2004), including insulin (Tillmar

et al., 2002), thereby increasing their stability and translation. Transcriptional mechanisms are critical for glucose regulation of insulin gene expression over a longer period of time. Major transcription factors that enhance insulin expression include Beta2/NeuroD, PDX-1, and RIPE3b1-Act/C1 (Melloul et al., 2002). Although the precise mechanism by which ICA512-CCF increases insulin gene expression remains to be defined, both its nuclear localization and its interaction with the SUMO-ligase PIASy suggest that it modulates transcription. Interestingly, sumoylation of PDX-1 enhances its stability, nuclear localization, and induction of insulin gene transcription (Kishi et al., 2003). In contrast, binding of RIPE3b1-Act/C1 to the C1 element in the insulin promoter is regulated by tyrosine phosphorylation (Zhao et al., 2000; Matsuoka et al., 2001). Thus, it would be interesting to test whether ICA512-CCF enhances insulin transcription by promoting the sumoylation of PDX-1 by PIAS proteins and/or by preventing the dephosphorylation of RIPE3b1-Act/C1 through its inactive PTP domain. An additional effector of ICA512-CCF could be STAT-5, which stimulates insulin gene expression and β -cell proliferation upon stimulation with growth hormone and prolactin (Nielsen et al., 2001). Interestingly, STAT-5 is activated by tyrosine phosphorylation (Herrington et al., 2000), whereas it is inhibited by PIAS3 (Ryeczyn and Clevenger, 2002). Moreover, induction of insulin gene expression by growth hormone depends on Ca^{2+} uptake, as it is prevented by verapamil, which blocks voltage-gated Ca^{2+} channels (Billestrup et al., 1995). Besides verapamil (German et al., 1990), other agents that block voltage-gated Ca^{2+} channels, such as D600 (Efrat et al., 1990) and nimodipine (Leibiger et al., 1998), were found to inhibit glucose-stimulated insulin gene expression in islets and insulinoma cells. However, these findings are still debated in view of seemingly discordant results by others (Goodison et al., 1992; de Vargas et al., 1997; Kennedy et al., 1999).

The effect of Ca^{2+} entry on insulin transcription has been partly attributed to its induction of insulin secretion (Leibiger et al., 1998). According to this interpretation, insulin acts as an autocrine signal that promotes its own gene expression through the activation of pathways downstream of insulin receptors in β -cells. However, more recent analyses have suggested that insulin receptors control islet development and the secretion (Kulkarni et al., 1999; Otani et al., 2004) but not the expression (Kennedy et al., 1999; Wicksteed et al., 2003) of insulin. Our findings point to the activation of μ -calpain and the generation of ICA512-CCF as a means by which Ca^{2+} can support glucose in inducing insulin gene expression.

Similar to ICA512, proteolytic cleavage of receptor proteins such as Notch and the Alzheimer's disease amyloid precursor protein (APP) generate intracellular fragments that are targeted to the nucleus and regulate gene expression (Fortini, 2002). The analogy of ICA512 with APP extends further, as processing of the latter is also modulated by calpain (Chen et al., 2004) and the cytoplasmic tail of both proteins binds PDZ-domain-containing proteins (Borg et al., 1998; Tomita et al., 1999; Ort et al., 2000). As discussed earlier in this section, binding of β 2-syntrophin could prevent ICA512-TMF cleavage and thereby its effect on gene expression. Likewise, the cy-

tosolic tail of APP binds the phosphotyrosine binding domain of Mints/X11s (Borg et al., 1998; Tomita et al., 1999), whose COOH-terminal PDZ domains, in turn, regulate the processing of APP and its transcriptional activity (Biederer et al., 2002). However, the case of ICA512 is unique as its cleavage is directly coupled in time and space to the exocytosis of SGs. Moreover, ICA512 is processed by μ -calpain without an involvement of γ -secretase, as it is the case for Notch and APP.

In conclusion, we propose a mechanistic model whereby cleavage of ICA512 upon SG exocytosis and translocation of its cytoplasmic tail to the nucleus allow β -cells to monitor their secretory activity in response to stimulation and to adjust their expression of insulin, and conceivably that of other genes, accordingly. As ICA512 is associated with SGs in virtually all neuroendocrine cells, this mechanism could be of general relevance for peptide hormone and neuropeptide secretion.

Materials and methods

Islet isolation and cell culture

Pancreatic islets from female Wistar rats were isolated and cultured (400/60 mm culture dish) as described previously (Gotoh et al., 1985). INS-1 cells were grown as described previously (Asfari et al., 1992).

cDNA cloning and transfection

Human ICA512 and ICA512(659–979) cDNAs were subcloned into pEGFP-N1 or pECFP-N1 (CLONTECH Laboratories, Inc.) as EcoRI–AgeI or AgeI–BglII inserts in frame with GFP or CFP, respectively. ICA512 was tagged at its COOH terminus with 3-HA epitopes by subcloning its cDNA as a KpnI–XbaI insert into the MoHa3 vector (a gift from M. Suchanek, Max Planck Institute of Molecular Cell Biology and Genetics [MPI-CBG], Dresden, Germany). Plasmids encoding proteolytically active and inactive tetanus toxin light chain were a gift from T. Binz (Medizinische Hochschule Hannover, Hannover, Germany; McMahon et al., 1993). The cDNAs of rat dynamin 1 and 2 and their corresponding dominant-negative (K44A) alleles fused to GFP were a gift from K. Simons (MPI-CBG, Dresden, Germany). Murine PIASy cDNA (a gift from R. Grosschedl, University of Munich, Munich, Germany) was inserted as EcoRI–BglII insert into pEYFP-N2 (CLONTECH Laboratories, Inc.). The cDNAs for lsm4-CFP and lsm4-YFP were a gift from K. Neugebauer (MPI-CBG, Dresden, Germany). The GeneStorm clone for human μ -calpain-V5 was purchased (Invitrogen). 4×10^6 INS-1 cells were electroporated with an Amaxa nucleoprep (Amaxa Biosystems) using the nucleofector kit V and the program T-20 (transfection efficiency ~50%). Electroporated cells were aliquoted in two 35-mm wells and grown for 4 d before harvesting. INS-1 cells stably expressing ICA512-GFP were selected with 600 μ g/ml neomycin and cloned by limiting dilution. Two clones expressing ICA512-GFP, as assessed by Western blotting and confocal microscopy, were selected for FACS using GFP fluorescence and further characterized.

Cell extraction and Western blotting

Unless otherwise stated, INS-1 cells and rat pancreatic islets (350–450) were preincubated for 1 h in resting buffer (0 mM glucose and 5 mM KCl) and then for various times in fresh resting or stimulating (25 mM glucose and 55 mM KCl or 25 mM glucose alone) buffer as described previously (Ort et al., 2001; Knoch et al., 2004). The protease inhibitors calpeptin, MG-132, and L-685,458 (Calbiochem) were added in the last 15 min of the preincubation, and then for the entire following period before cells were harvested. Cells were washed with ice cold PBS and extracted in lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 1% protease inhibitor cocktail [Sigma-Aldrich]) at 4°C. Cytosolic and nuclear extracts were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical Co.), according to the manufacturer's protocol. Protein concentration was assessed with the Protein Assay Reagent (Bio-Rad Laboratories). Proteins were separated by 8–10% SDS-PAGE, transferred on nitrocellulose filters, and detected with the following antibodies: mouse monoclonals anti-ICA512 (Hermel et al., 1999), anti- μ -calpain (Chemicon), anti- γ -tubulin and anti-HA (Sigma-Aldrich), and anti-GFP (CLONTECH Laboratories, Inc.); rabbit polyclonals anti-Glut2 (Alpha Diagnostics International), anti-

CPE (Chemicon), anti-GFP (Molecular Probes), and anti-HA (a gift from C. Thiele, MPI-CBG, Dresden, Germany); and anti-V5-HRP (Invitrogen). Protein signals were detected by chemiluminescence with the Supersignal West Pico Substrate (Pierce Chemical Co.) using a LAS 3000 Bioimaging System (Fuji) and quantified with the Image Gauge v3.45 software (Fuji).

Pulse-chase labeling and immunoprecipitation

4×10^7 INS-1 cells grown in culture for 4 d were starved for 1 h in methionine-free labeling medium with 5.5 mM glucose (BioConcept), and then pulsed with 800 μ Ci [35 S]methionine in the same buffer for 1 h at 19°C to prevent the exit of newly synthesized secretory proteins, including pro-ICA512, from the trans-Golgi network. Next, cells were either stimulated for 30, 60, or 120 min or kept at rest for 120 min at 37°C and extracted in lysis buffer. 3.6 mg of protein from each lysate was incubated for 90 min with 60 μ l of 50% slurry protein G-sepharose (Amersham Biosciences) in 900 μ l for precleaning. After centrifugation, each supernatant was split in two aliquots, which were incubated overnight either with a rabbit affinity-purified antibody directed against the juxtamembrane domain of ICA512 (Hermel et al., 1999) or with a mouse monoclonal anti-synaptophysin antibody (Synaptic Systems GmbH). Protein G-sepharose beads were added the next day for 90 min, centrifuged and washed eight times with 1 ml of lysis buffer, and finally resuspended and boiled in SDS-sample buffer. Proteins were separated by 8% SDS-PAGE and transferred on nitrocellulose. Autoradiographies of the filters were collected with a phosphorimager (model BAS 1800 II; Fuji) after 2.5 d of exposure, and processed with Image Reader V1.8 (Fuji). The same filters were immunoblotted with mouse monoclonal anti-ICA512 or anti-synaptophysin antibodies.

Time course stimulation

INS-1 cells were incubated for 1 h in resting buffer (2.8 mM glucose and 5 mM KCl) and for 6 h in resting or stimulating buffer, with or without 40 μ M calpeptin, and then again in resting buffer for up to 24 h. Total RNA for real-time PCR was collected at time 0, 2, and 6 h during stimulation and at 2, 6, 12, and 24 h during the following resting period.

Sucrose density gradient fractionation

INS-1 and INS-1 ICA512-GFP cells were fractionated on continuous sucrose density gradients from 0.4–2 M as described previously (Ort et al., 2000). Fractions were separated by SDS-PAGE and analyzed by immunoblotting as indicated in Cell extraction and Western blotting.

GST pull-down assays

ICA512(601–979) in pET28a (Ort et al., 2000) and PIASy in pcDNA3.1 (Invitrogen) were transcribed and translated in vitro with the T7/coupled transcription-translation system (Promega). PIAS1-GST, PIASy-GST, GST-p53, and GST-ICA512(601–979) were expressed in bacteria as described previously (Ort et al., 2000; Sachdev et al., 2001; Schmidt and Muller, 2002). [35 S]methionine-labeled ICA512(601–979) or PIASy were incubated overnight at 4°C in GST binding buffer (120 mM NaCl, 50 mM Tris, pH 8, 0.25% NP-40, 1 mM DTT, and 1 mM PMSF) with GST-fusion proteins or GST alone coupled to glutathione-sepharose beads (Amersham Biosciences). Beads were washed five times with binding buffer and eluted with 10 mM of reduced glutathione. The eluate was subjected to SDS-PAGE and analyzed by autoradiography.

In vitro μ -calpain cleavage of ICA512

3 μ g of bacterially expressed His-ICA512(601–979) was digested with μ -calpain (Calbiochem), with or without 30 μ M calpeptin or MG-132, as described previously (Ort et al., 2001).

Immunocytochemistry

INS-1 cells in resting or stimulated buffers were fixed with 2% PFA, permeabilized with 0.2% saponin, and immunostained with mouse monoclonal anti- μ -calpain and insulin (Sigma-Aldrich) or rabbit polyclonal anti-ICA512ecto (Solimena et al., 1996) antibodies for 1 h, followed by goat anti-mouse Alexa⁴⁸⁸ or Alexa⁵⁶⁸ conjugated IgGs (Molecular Probes). Nuclei were counterstained with DAPI (Sigma-Aldrich). 0.5- μ m Z-section images were acquired with an inverted 405-nm confocal microscope (model LSM Meta; Carl Zeiss Microimaging, Inc.).

EM

Cells were fixed with 4% PFA in 60 mM Pipes, 25 mM Hepes, 10 mM EDTA, and 2 mM MgCl₂, pH 6.9, for 1 h at RT and scraped and pelleted in PBS with 10% gelatin. Pellets were fixed and stored in PBS with 1% PFA. Ultrathin cryosections were prepared as described previously (Moonen et al., 1997) and incubated with mouse anti-insulin (Sigma-Aldrich;

1:1,000) and rabbit anti-GFP (Molecular Probes; 1:500) antibodies followed by goat anti-mouse and goat anti-rabbit IgGs conjugated to 6 nm of gold and 12 nm of gold particles, respectively (Dianova; 1:30). Specificity of the GFP-labeling on granules was assessed by morphometry in two independent experiments.

FRET

INS-1 cells transiently cotransfected with ICA512-CFP, PIAS-γ-YFP, Ism4-YFP, and Ism4-CFP were kept at rest or stimulated for 90 min. FRET in 2% PFA fixed cells was assessed by measuring the dequenching of CFP during the photobleaching of YFP performed with the 514-nm laser line at 70% power and for six cycles of 30 s each. Mean fluorescence of CFP and YFP were determined by tracing the nuclear perimeter using the "region of interest" software function. Fluorescence intensity of CFP after photobleaching was normalized to 100% at time 0. Six to nine fields for each slide were photobleached and recorded. Each experiment was independently performed two times.

Transferrin uptake assay

INS-1 cells transfected with dynamin-GFP constructs were starved for 1 h in medium without FBS and incubated for 15 min in serum-free medium with Alexa⁵⁶⁸-transferrin (Molecular Probes) and 1 mg/ml BSA on 37°C. Cells were washed 4× in cold PBS and fixed as indicated in the section Immunocytochemistry. Images were acquired with a CCD camera (CoolSnap-HQ; Roper Scientific) attached to a microscope (model BX61; Olympus) and processed with Metamorph 4.65 software (Universal Imaging Corp.).

RNA interference

INS-1 cells (6×10^5 /35-mm well) were grown for 2 d before transfection. 21-mer silencing RNA (siRNA) oligonucleotides 1, 2, and 3 for rat μ -calpain were synthesized with the Silencer siRNA Construction Kit (Ambion) using the following primers: sense 1, 5'-AAGAGAGCGCTCCAGAACTCGCTGTCTC-3'; antisense 1, 5'-AACGAGTCTGGAGCGCTCTCCCTGTCTC-3'; sense 2, 5'-AACCTGGATCTGGTCATCTAGCCTGTCTC-3'; antisense 2, 5'-AAGTAGATGACCAAGATCCAGGCCTGTCTC-3'; sense 3, 5'-AAGTACTGACCTCTCGAAGGCCTGTCTC-3'; antisense 3, 5'-AACCTTCGAGAGGTCAAGCAACCTGTCTC-3'. Cells were transfected on day 2 and 4 with 1 μ g of siRNA oligos per well in OptiMEM medium with Lipofectamine (Invitrogen). On day 6, cells were harvested and processed for RT-PCR, real-time PCR, or immunoblotting.

Firefly luciferase assay

2 d before transfection of siRNA oligos for firefly luciferase (Knoch et al., 2004), INS-1 cells were electroporated with pGL3-Basic and pRL vectors (Promega) for coexpression of firefly and renilla luciferase, respectively. Firefly luciferase expression and activity were measured 6 d after plating of the cells.

Insulin RIA

Cells were extracted overnight in 3:1:0.06 volumes of ethanol, H₂O, and 37% HCl at -20°C. Insulin content in the cells and in the medium was measured with the Sensitive Rat Insulin RIA Kit (Linco Research). The stimulation index was calculated as follows: secreted insulin:cell insulin content + secreted insulin from stimulated cells/secreted insulin:cell insulin content + secreted insulin from resting cells. The stimulation index from control cells was equaled to 100%.

RT-PCR

Cytosolic RNA was isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. 1 μ g of RNA was used for the simultaneous amplification of rat μ -calpain and β -actin cDNAs by RT-PCR using the Titan One Tube RT-PCR System (Roche Diagnostics) and 0.4 μ M primers for each gene. Primers for μ -calpain were as follows: sense, 5'-AACGAGTCTGGAGCGCTCTC-3'; antisense, 5'-CTTCAGAATGATCTGGTAGAGG-3'. The number of cycles had been optimized to be within the logarithmic phase of the reaction. PCR products were separated on 2% agarose gels, stained with ethidium bromide, and semiquantitated with the GeneSnap software (Syngene) following image acquisition with GeneGnome (Syngene).

Real-time PCR

Total RNA from INS-1 cells was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. 1 μ g of total RNA was used for reverse transcription with 2 μ M of specific antisense primers for insulin and β -actin, as described previously (Steinbrenner et al., 2002). mRNA expression was measured by quantitative real-time PCR using the Brilliant

SYBR Green QPCR (Stratagene) with an Mx4000 Multiplex Quantitative Real Time PCR System (Stratagene).

Online supplemental material

Fig. S1 shows that ICA512 is a substrate of μ -calpain cleavage both in vitro and within cells. Fig. S2 shows that dynamin 2 (K44A)-GFP inhibits the uptake of transferrin in INS-1 cells. Fig. S3 shows that GFP is not enriched in the nuclei of stimulated INS-1 cells. Fig. S4 shows that ICA512-HA3 is correctly processed and targeted to the nucleus of stimulated INS-1 cells. Fig. S5 is of FRET images showing that ICA512-CFP does not interact with the control protein Ism4-YFP in the nucleus of stimulated INS-1 cells. Video 1 is a stack of serial optical confocal Z-sections showing the low levels of ICA512-GFP in the nuclei of resting INS-1 cells. Video 2 is a stack of serial optical confocal Z-sections showing the high levels of ICA512-GFP in the nuclei of stimulated INS-1 cells. Video 3 is a stack of serial optical confocal Z-sections showing the low levels of ICA512-HA3 in the nuclei of resting INS-1 cells. Video 4 is a stack of serial optical confocal Z-sections showing the high levels of ICA512-HA3 in the nuclei of stimulated INS-1 cells. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200408172/DC1>.

M. Solimena dedicates this work to his daughters.

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