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ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules

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Islet cell autoantigen (ICA) 512 is a novel autoantigen of insulin-dependent diabetes mellitus (IDDM) which is homologous to receptor-type protein tyrosine phosphatases (PTPases). We show that ICA 512 is an intrinsic membrane protein of secretory granules expressed in insulin-producing pancreatic β-cells as well as in virtually all other peptide-secreting endocrine cells and neurons containing neurosecretory granules. ICA 512 is cleaved at its luminal domain and, following exposure at the cell surface, recycles to the Golgi complex region and is sorted into newly formed secretory granules. By immunoprecipitation, anti-ICA 512 autoantibodies were detected in 15/17 (88%) newly diagnosed IDDM patients, but not in 10/10 healthy subjects. These results suggest that tyrosine phosphorylation participates in some aspect of secretory granule function common to all neuroendocrine cells and that a subset of autoantibodies in IDDM is directed against an integral membrane protein of insulin-containing granules.

Keywords: autoantigens/insulin-dependent diabetes mellitus/1A-2/neurosecretory granules/protein tyrosine phosphatase

Introduction

Insulin-dependent diabetes mellitus (IDDM) results from the autoimmune destruction of pancreatic β-cells, the cells responsible for insulin production and secretion. Lymphocytic infiltrates, in which CD8+ T-cells predominate, are present in the islets of newly diagnosed IDDM patients as well as in cases of recurrent diabetes following pancreas isograft between human leukocyte antigen (HLA)-identical twins (Bottazzo *et al.*, 1985; Sibley *et al.*, 1985). Likewise, insulitis precedes the β-cell destruction in the two spontaneous animal models of IDDM, the non-obese diabetic (NOD) mouse and the bio-breeding (BB) rat. CD4+ and CD8+ T-cells can adoptively transfer the disease in rodent models (Miller *et al.*, 1988; Reich *et al.*, 1989). Anti-T-cell agents such as monoclonal antibodies directed against CD4, CD8, intercellular cell adhesion molecule (ICAM)-1 or major histocompatibility complex (MHC) molecules, immunosuppressive drugs and neonatal thymectomy can prevent diabetes in NOD mice and BB rats. Furthermore, the development of the disease in humans as well as in NOD mice is associated strongly with the MHC of class II genes. Since the only known function of class II MHC molecules is to present peptides to T-cells, these data further suggest that IDDM is caused by T-cells infiltrating pancreatic islets and directly destroying pancreatic β-cells.

Despite much effort, our knowledge of the β-cell autoantigens recognized by pathogenic T-cells is limited. There are data indicating an involvement of β-cell secretory granule antigens in the pathogenesis of IDMM. Insulin and carboxypeptidase H, two target autoantigens of IDDM autoantibodies, are both localized in the β-cell secretory granules. T-cells from IDDM patients proliferate when challenged with membrane antigens enriched in insulin-containing secretory granules, including an as yet uncharacterized 38 kDa protein (Roep *et al.*, 1990, 1995). T-cell clones isolated from NOD mice also react against secretory granule antigens from NOD insulinomas (Bergman and Haskins, 1994). However, all IDDM autoantigens characterized thus far, including insulin (Palmer *et al.*, 1983), glutamic acid decarboxylase (GAD) (Bækkeskov *et al.*, 1990), carboxypeptidase H (Castano *et al.*, 1991), islet cell autoantigen (ICA) 69 (Pietropaolo *et al.*, 1993) and ICA 512 have been originally identified as the targets of IDDM patient autoantibodies. It is not yet clear whether autoantibodies to β-cell antigens contribute to the development of the disease or simply represent an epiphenomenon that follows β-cell destruction. On the other hand, T-cells isolated from IDDM patients proliferate in response to insulin (Keller, 1990; Harrison *et al.*, 1991) and GAD (Atkinson *et al.*, 1994; Panina-Bordignon *et al.*, 1995), suggesting that protein targets of patient autoantibodies may also be relevant T-cell autoantigens (Solimena and De Camilli, 1994).

ICA 512, a new β-cell autoantigen of IDDM, was cloned recently by screening a human islet cDNA expression library with a pool of IDDM patient sera (Rabin *et al.*, 1992, 1994). Independently, a search for clones enriched in a human insulinoma cDNA library obtained by subtracting glucagonoma from insulinoma cDNAs (Lan *et al.*, 1994) led to the identification of the same protein, which in this case was defined as IA-2. Subsequent analysis of ICA 512 clones showed that the published discrepancies between ICA 512 and IA-2 were due to technical factors and that the cDNA sequence of ICA 512 is identical to IA-2 within the coding region; thus the two proteins are identical (henceforth defined as ICA 512). According to the predicted amino acid sequence, ICA 512

is an intrinsic membrane protein of 979 amino acids with a single transmembrane region encompassing residues 577–601 and with significant homology to receptor type-protein tyrosine phophatases (RT-PTPases). Interestingly, the predicted protein structure of its cytoplasmic region contains only one PTPase domain, like HPTPβ, rather than two, as is more commonly found in RT-PTPases (Krueger *et al.*, 1990; Saito *et al.*, 1992). Neither human (Lan *et al.*, 1994; Rabin *et al.*, 1994) nor mouse (Lu *et al.*, 1994) recombinant ICA 512 expressed in bacteria had tyrosine phosphatase activity when tested with several common PTPase substrates. Northern blot experiments have shown that, in addition to pancreatic islets, ICA 512 is expressed in brain and pituitary, but not in a variety of non-neuroendocrine tissues.

In the present study we demonstrate that ICA 512 is an intrinsic membrane protein of secretory granules. We show evidence indicating that the protein undergoes proteolytic cleavage at its luminal domain and that, upon exocytosis of secretory granules, ICA 512 recycles to the Golgi complex region to be sorted into newly formed secretory granules. Because of the virtually ubiquitous expression of ICA 512 in neuroendocrine cells and its homology to RT-PTPases, we suggest that tyrosine phosphorylation is likely to play a role in the life-cycle of secretory granules. We also show that 15/17 (88%) newly diagnosed IDDM patients have autoantibodies directed against bovine ICA 512, demonstrating that ICA 512 is a dominant autoantigen of IDDM and adding to the growing evidence that proteins associated with β-cell secretory vesicles are the preferential targets of autoimmunity in IDDM.

Results

Expression of ICA 512 is restricted to neuroendocrine cells

As a first step in the study of the cell biology of ICA 512, we investigated by immunocytochemistry its tissue distribution in the rat (Figure 1). In general, the patterns of immunoreactivity obtained with the two rabbit antisera directed against either the cytoplasmic (89-59) or the luminal (92-18) domain of human ICA 512 were identical. Double immunofluorescence with anti-glucagon and anti-somatostatin antibodies on pancreatic tissue sections showed that ICA 512 is present in β-, α- and δ-cells (Figure 1a–d). Other neuroendocrine tissues expressing ICA 512 included the anterior, intermediate and posterior pituitary, as well as the adrenal medulla (Figure 1g, h and j). Conversely, ICA 512 was not detected in endocrine cells secreting hydrophobic hormones such as the cells of the adrenal cortex (Figure 1g) and the principal cells of the thyroid (not shown), neither of which contain secretory granules. Similarly, no detectable ICA 512 expression was observed in non-endocrine cells including acinar pancreatic cells (Figure 1a–e), gastric parietal cells (Figure 1h), hepatocytes (Figure 1i) or striated muscle and spleen cells (not shown). In all peripheral tissues examined, however, ICA 512 immunoreactivity was detectable in autonomic nerve fibers and ganglia (Figure 1e, f and h). This immunoreactivity was particularly intense at synaptic contacts, as shown by its partial co-localization with synapsin I (Figure 1e and f), a marker of neuronal pre-synaptic terminals (De Camilli *et al.*, 1990).

In brain, high levels of ICA 512 immunoreactivity were detected in the amygdala and hypothalamus (Figure 1k and l). Because they are enriched in secretory granules compared with other parts of the central nervous system, the amygdala and hypothalamus are regarded as the neuroendocrine regions of the brain. In these regions ICA 512 immunoreactivity was detected in neuronal cell bodies, axons and nerve endings, but not in dendrites, suggesting that ICA 512 is enriched in pre-synaptic terminals, where both secretory granules and synaptic vesicles are concentrated. The overall distribution of ICA 512, however, was unlike that for synaptic vesicle markers such as synapsin I, which are detectable in every brain region. In particular, ICA 512 immunoreactivity was below detectable levels in the cerebral cortex (Figure 1n), hippocampus, cerebellar cortex, striatum and thalamus (not shown). All these regions contain considerably fewer secretory granules than the amygdala and hypothalamus. The highest levels of ICA 512 immunoreactivity were observed in the infundibular tract and posterior pituitary (Figure 1j). The infundibular tract contains axons originating from hypothalamic neurons which project to the posterior pituitary, the organ which contains the highest concentration of secretory granules in the entire body.

In summary, ICA 512 was detected exclusively in peptide-secreting endocrine cells, in neurons of the autonomic nervous system and in neuroendocrine neurons of the brain, all of which contain neurosecretory granules.

ICA 512 co-localizes with neurosecretory granules in cultured cells

We next performed immunocytochemistry on cells in culture, a procedure which provides a more defined resolution of the intracellular localization of an antigen than antibody staining of tissue sections (Figure 2). In 5-day-old primary cultured rat hypothalamic neurons, ICA 512 immunoreactivity was restricted to neuronal perikaria and axonal processes, whereas dendrites and the underlying glial cells were unstained (Figure 2a–c). Within axons, ICA 512 immunoreactivity was prominent at pre-synaptic varicosities, where secretory granules are concentrated. Conversely, ICA 512 was not detected in 10-day-old primary cultured rat hippocampal neurons (not shown), consistent with the lack of ICA 512 immunoreactivity in the hippocampus found by *in situ* immunocytochemistry. In rat insulinoma (RIN) cells, ICA 512 immunoreactivity was particulate and co-localized with insulin in the area of the Golgi complex and near the cell surface (Figure 2d–f). On the other hand, ICA 512 did not co-localize with transferrin receptor, a marker of constitutively exocytosing membranes, nor with synaptophysin or lgp 120, two marker proteins of synaptic-like microvesicles and lysosomes, respectively (not shown). No surface staining was detected when 4% paraformaldehyde-fixed, non-permeabilized RIN cells were immunolabeled with the 92-18 serum, which is directed against the ectodomain of ICA 512 (not shown). Taken together, these data suggest that ICA 512 is associated with secretory granules and it is not a resident protein of the plasma membrane.

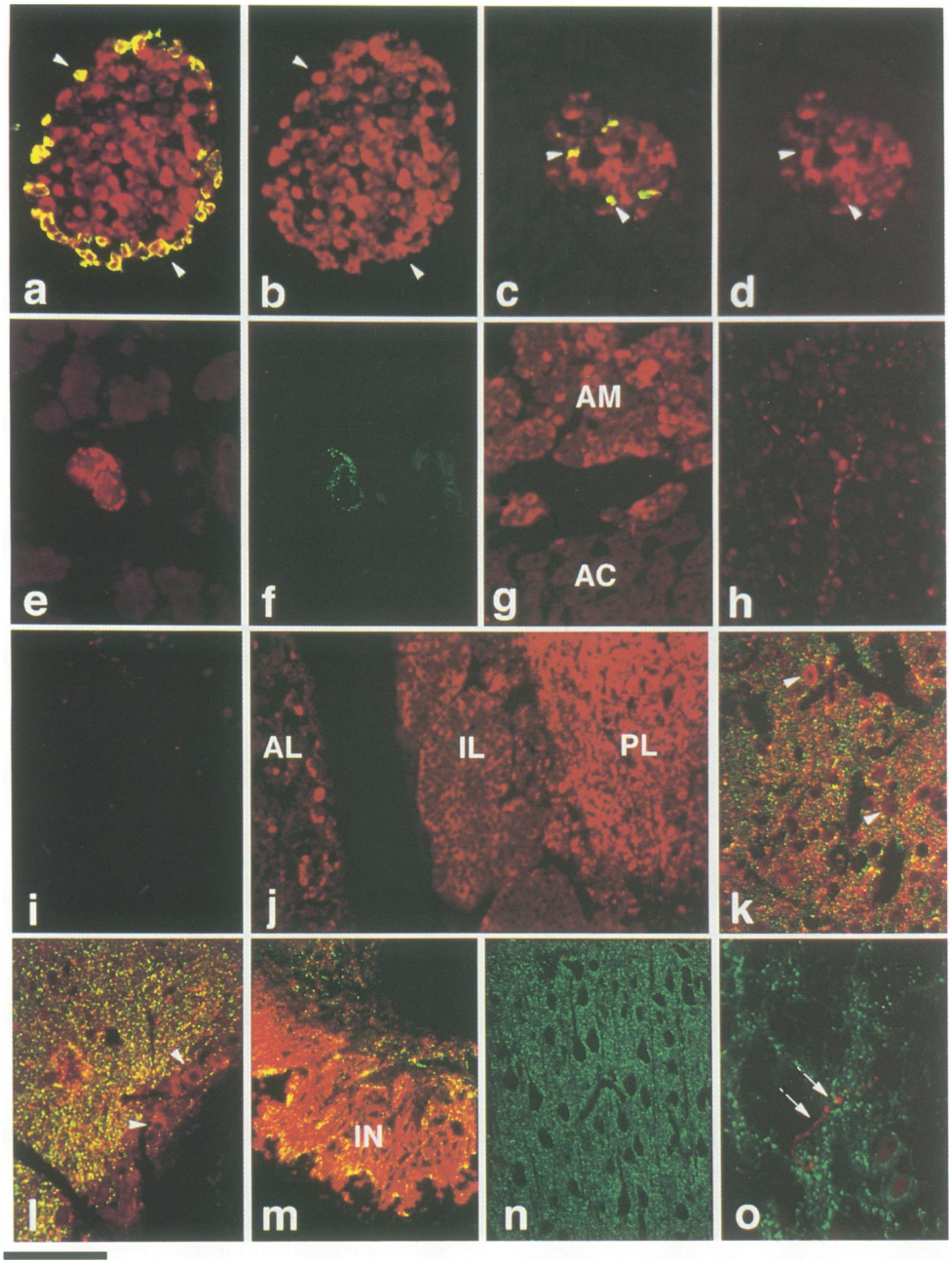

Fig. 1. Distribution of ICA 512 in rat tissues by immunocytochemistry and confocal microscopy. ICA 512 immunoreactivity (pseudocolor red) was detected with rabbit 89-59 (a, b, f, g, j and m) or 92-18 sera (c, d, h, i, l, n and o). Double immunostaining was performed with mouse anti-glucagon, anti-somatostatin, anti-synapsin or anti-GAD65 antibodies (pseudocolor green). Co-localization of ICA 512 with glucagon, somatostatin, synapsin or GAD65 results in orange–yellow pseudocolors. (**a–d**) ICA 512 is expressed in all pancreatic islet cells, including β-cells, but not in the exocrine pancreas. (a and c) Double staining for ICA 512, glucagon (a) or somatostatin (c). (b and d) Red channels of (a) and (c), respectively, showing the ICA 512 immunoreactivity alone. Arrowheads point to α- (a and b) and δ- (c and d) islet cells expressing ICA 512. (**e** and **f**) Partial co-localization of ICA 512 (e) and synapsin (f) in nerve terminals of the autonomic nervous system in the pancreas. ICA 512 immunoreactivity is also detected in the cell body of ganglionic neurons (e). (**g–i**) ICA 512 is expressed in chromaffin cells of the adrenal medulla (g, AM), autonomic fibers in the gastric mucosa (h, arrows), but not in the adrenal cortex (g, AC), gastric epithelial cells (h) and liver (i). (**j**) In the pituitary ICA 512 immunoreactivity is most prominent in the posterior lobe (PL), but is also detected in the cells of the anterior (AL) and intermediate (IL) lobes. (**k–o**) In brain ICA 512 is concentrated in the amygdala (k), hypothalamus (l) and infundibular tract (i, IN) whereas in other regions, such as the cerebral cortex (n) and brainstem (**o**) ICA 512 levels are very low. (k), (m) and (n) show double labelings for ICA 512 and synapsin; (l) and (o) show the double labeling for ICA 512 and GAD65. Arrowheads point to the cell bodies of ICA 512-positive neurons in the amygdala (k) and supraoptic nuclei (l). ICA 512 has a particulate distribution and partially co-localizes with synapsin and GAD65, consistent with its accumulation at axonal varicosities (o, arrows) and nerve terminals. Scale bar is 125 μm for (a–n) and 65 μM for (o).

ICA 512 is associated with regulated secretory vesicles

If ICA 512 is an intrinsic membrane protein of neurosecretory granules, its accessibility to antibodies directed against its luminal domain should be increased upon stimulation of secretory granule exocytosis. We tested this hypothesis by immunocytochemistry on RIN cells incubated for 2 min or 10 min in resting (1 mM KCl) or stimulating

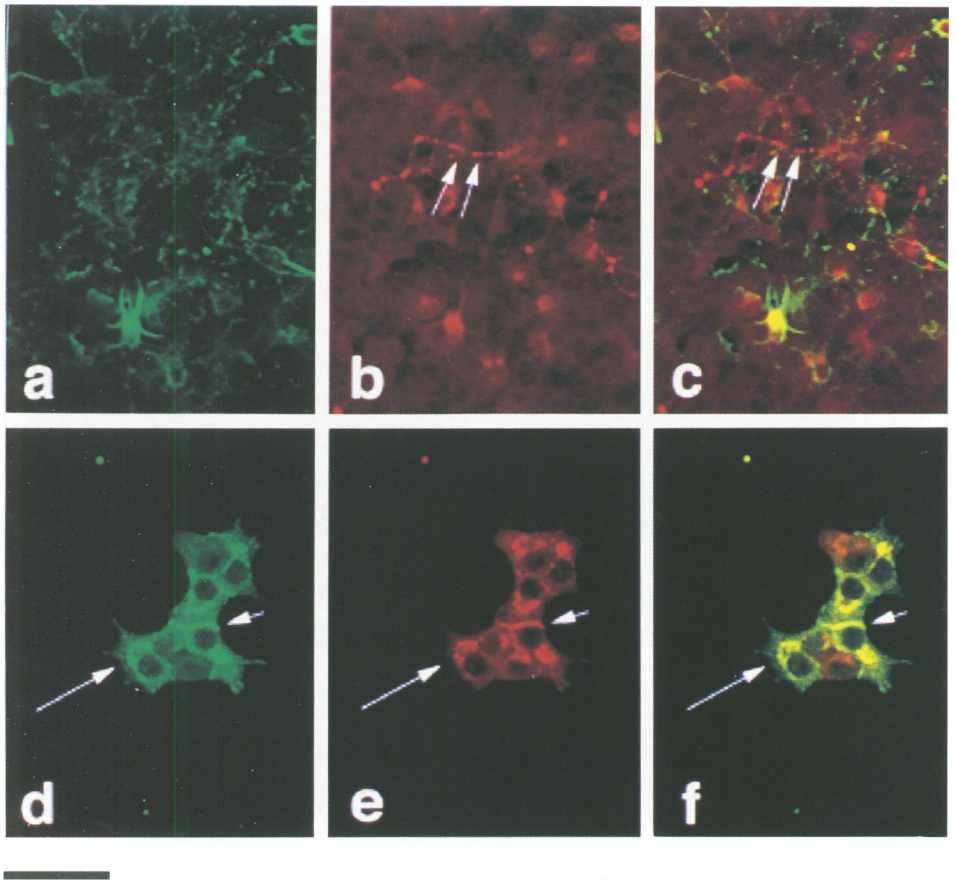

Fig. 2. Intracellular distribution of ICA 512 by confocal microscopy in rat hypothalamic neurons (a–c) and rat insulinoma cells (d–f). (**a–c**) Hypothalamic cell cultures double stained for the dendritic marker MAP2 (green pseudocolor) and ICA 512 (red pseudocolor). (a and b) Green and red channels of the image shown in (c), respectively. ICA 512 is present in axonal varicosities (arrows in b and c), and neuronal cell bodies, but not in dendrites. (**d–f**) RIN cells double stained for insulin (green pseudocolor) and ICA 512 (red pseudocolor). (d and e) Green and red channels of the image shown in (f), respectively. ICA 512 co-localizes with insulin (f, orange–yellow pseudodocolor), both in the area of the Golgi complex (long arrows) as well as in the proximity of the plasma membrane (short arrows). All cells express ICA 512, but a few contain very low levels of insulin. Scale bar is 100 μm for (a–f).

(55 mM KCl) buffer in the presence of the anti-ICA 512 ectodomain antibodies. No immunoreactivity was detectable in RIN cells exposed to anti-ICA 512 ectodomain antibodies for 2 min in either unstimulated or stimulated conditions (not shown). However, a prominent staining of the plasma membrane region was detected in cells incubated for 10 min in stimulating conditions (Figure 3a), but not in cells incubated for the same length of time in resting conditions (Figure 3b). RIN cells exposed to the 92-18 pre-immune serum or anti-ICA 512 cytoplasmic domain antibodies were not immunolabeled (not shown). These data clearly show that exposure of ICA 512 at the cell surface is enhanced by treatments that trigger regulated exocytosis, further suggesting that the protein is associated with secretory granules.

Localization of ICA 512 on neurosecretory granules by immunoelectron microscopy

The intracellular localization of ICA 512 was investigated further by immunoelectron microscopy on ultrathin frozen sections of rat posterior pituitary (Figure 4). A prominent immunogold labeling of neurosecretory granules, but not of synaptic vesicles, was observed on the sections incubated with anti-ICA 512 89-59 and 92-18 antibodies (Figure 4a–d). In nerve endings of the posterior pituitary, ICA 512 immunoreactivity was also detected on some large clear vesicles which may originate from the retrieval of secretory granule membranes after exocytosis. Only background levels of gold particles were observed on other intracellular structures, including mitochondria, the plasma membrane of neurosecretosomes, or the cytoplasm and nuclei of pituicytes. As previously reported, anti-synaptophysin antibodies specifically immunolabeled synaptic vesicles (Navone *et al.*, 1989) (Figure 4e). Finally, only a few gold particles were detected on sections incubated with 89-59 or 92-18 pre-immune sera (Figure 4f and not shown). These data conclusively demonstrated that ICA 512 is localized on secretory granules.

Internalized ICA 512 recycles to newly formed secretory granules

What is the fate of ICA 512 following exposure at the cell surface? To address this question, RIN cells were incubated with anti-ICA 512 ectodomain antibodies in stimulating buffer (55 mM KCl) for 1 h and then were either immediately fixed and permeabilized or were allowed to recover for 14 h before fixation and permeabilization. Uptake of anti-ICA 512 ectodomain antibodies was then visualized with rhodamine-conjugated goat anti-rabbit IgG. In RIN cells fixed immediately after incubation, anti-

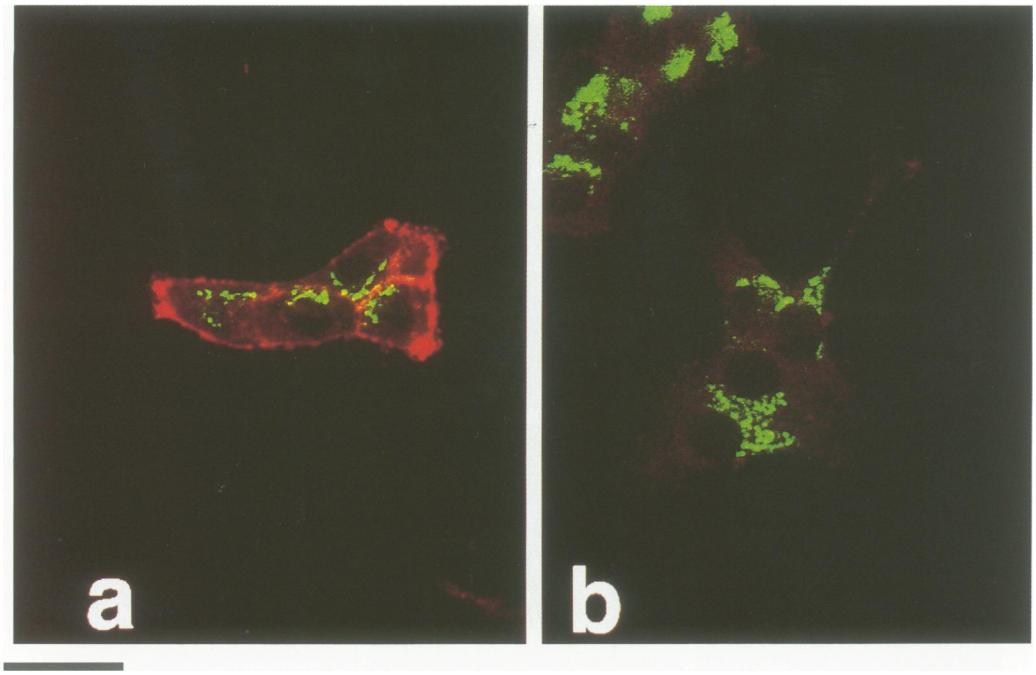

Fig. 3. Exposure of ICA 512 at the plasma membrane of RIN cells in resting and stimulating conditions. RIN cells incubated in 55 mM KCl (**a**) or 1 mM KCl (**b**) KRH buffer with anti-ICA 512 ectodomain antibodies for 10 min and counterstained after fixation with anti-transferrin receptor antibodies. A prominent immunolabeling of the plasma membrane region is visible in stimulated cells (a), but not in unstimulated cells (b). Scale bar is 50 μm for (a) and (b).

ICA 512 ectodomain antibodies were detected in close proximity to the plasma membrane as well as throughout the cytoplasm (Figure 5b and c; compare also with Figure 3a) and in a perinuclear region. This staining overlapped to some extent with transferrin receptor (Figure 5a and c), suggesting the internalization of ICA 512 in endosomal structures. After 14 h of recovery, anti-ICA 512 ectodomain antibodies were most abundant in the Golgi complex region (Figure 5h–i) as shown by its co-localization with insulin (Figure 5g and i). Furthermore, a pool of retrieved ICA 512 was also visible in insulin-containing secretory granules located at the cell periphery (arrows in Figure 5g–i). At this time point only, however, a limited overlap was observed between internalized anti-ICA 512 ectodomain antibodies and transferrin receptor (arrows in Figure 5d–f). These results demonstrate that, upon retrieval from the cell surface, ICA 512 recycles to the Golgi complex region and is sorted into newly formed secretory granules.

Anti-ICA 512 antibodies react with a 70 kDa intrinsic membrane protein

Immunocytochemistry indicates that ICA 512 is very abundant in the posterior pituitary (Figure 1). This organ, which is composed for the most part of the nerve endings of neurons of the supraoptic and paraventricular hypothalamic nuclei, secretes vasopressin and oxytocin into the blood stream. Both hormones are stored in neurosecretory granules which almost entirely fill the nerve endings of the posterior pituitary. Being large and well defined from the anterior pituitary, we used the bovine posterior pituitary as a source of material to investigate further the biochemical properties of ICA 512.

Both anti-ICA 512 rabbit antisera reacted with a broad protein band of ~70 kDa in a Western blot of post-nuclear supernatant from bovine posterior pituitary (Figure 6A, lanes 1 and 2). The same protein was also detected when these antibodies were affinity purified. A less abundant protein with an identical electrophoretic mobility was detected with both antibodies in total protein homogenates of human pituitary adenoma and mouse insulinoma (not shown). Neither antibody reacted with a protein in the expected size range of 106 kDa (predicted size of ICA 512 based on its primary amino acid sequence). Preincubation of 89-59 and 92-18 antisera with their respective ICA 512 recombinant immunizing fragments was sufficient to abolish their reactivity with the 70 kDa protein completely (not shown).

Upon subcellular fractionation, the 70 kDa protein was only recovered in the high speed pellet (Figure 6B, lanes 1 and 2). Washing of the high speed pellet with 1 M NaCl or with 0.1 M Na_2CO_3, pH 11.5 (two treatments which cause the dissociation of peripherally associated membrane proteins from membranes) did not affect the recovery of the 70 kDa protein in the high speed pellet (Figure 6B, lanes 3–6). The 70 kDa protein could only be extracted from membranes with detergent (Figure 6B, lanes 7–8). Furthermore, the only 89-59 reactive antigen immunoprecipitated by two anti-ICA 512 monoclonal antibodies from bovine posterior pituitary was a 70 kDa protein (Figure 6B, lanes 9–10). Thus, the 70 kDa protein recognized by anti-ICA 512 antibodies had the biochemical and immunological properties expected for ICA 512.

The 70 kDa protein recognized by anti-ICA 512 antibodies is enriched in secretory granules

To assess further the relationship between the 70 kDa protein recognized by anti-ICA 512 antibodies and ICA

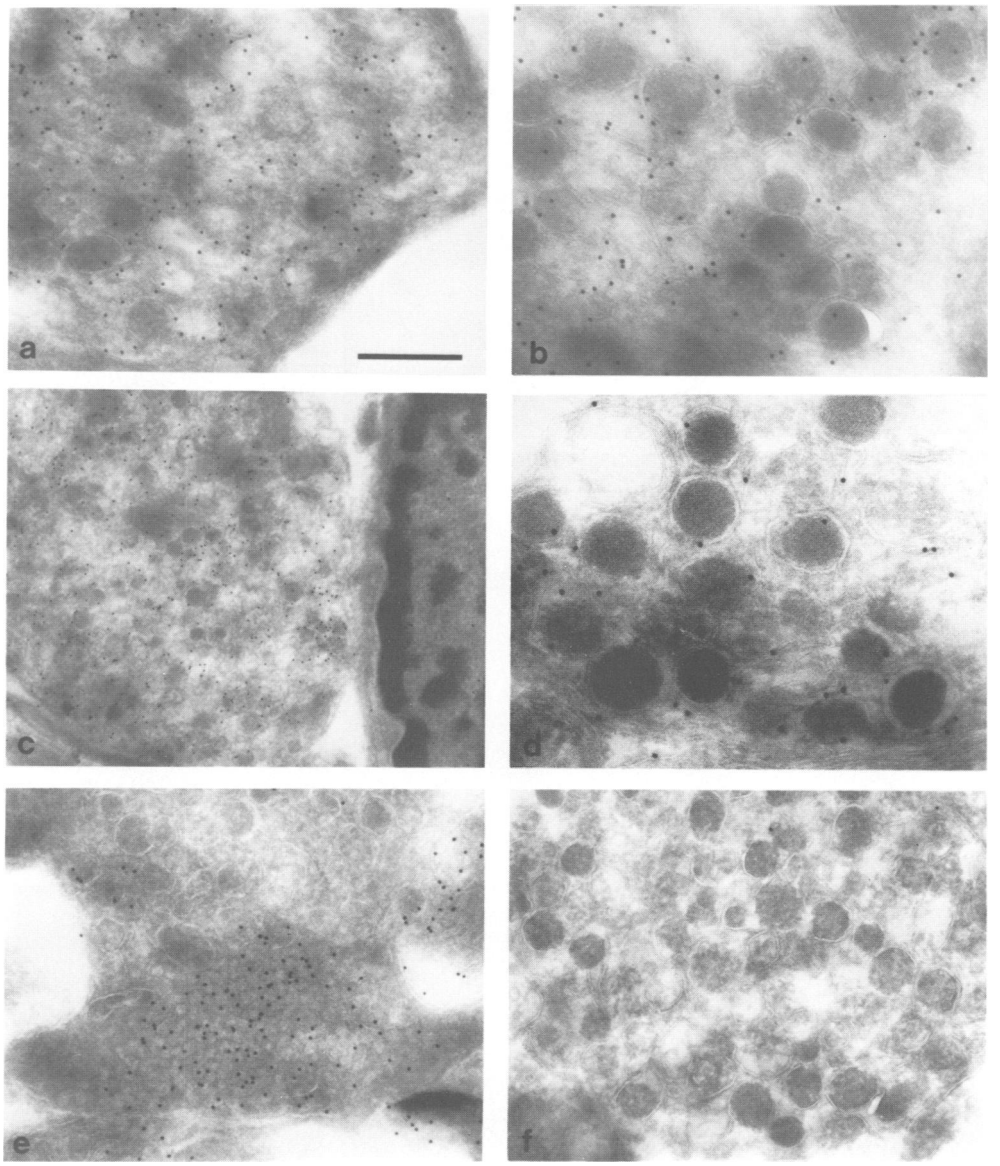

Fig. 4. Immunoelectron microscopy on ultrathin frozen sections of rat posterior pituitary. (**a** and **b**) Immunogold staining for ICA 512 with 89-59 anti-ICA 512 cytoplasmic domain serum. (**c** and **d**) Immunogold staining for ICA 512 with 92-18 anti-ICA 512 luminal domain serum. (**e**) Immunogold staining for the synaptic vesicle marker synaptophysin. (**f**) Control immunogold staining with 92-18 pre-immune serum. In neurosecretosomes ICA 512 immunoreactivity is primarily associated with 100–200 nm dense core vesicles which correspond to neurosecretory granules. Some immunogold particles are also present on the membranes of larger vesicles which do not have a dense core and which may represent secretory granule membranes retrieved after exocytosis. Virtually no immunolabeling for ICA 512 is present on neurosecretosome plasma membranes (a and c), as well as in the cytoplasm and nuclei of a pituicyte (c). For the purpose of comparison, the immunostaining for synaptophysin, which is almost exclusively associated with synaptic vesicles is shown in (e). No immunolabeling of neurosecretory granules is appreciable in sections incubated with 92-18 pre-immune serum (f). Scale bar in (a) is 293 nm for (a), (e) and (f); 226 nm for (b), 496 nm for (c), and 197 nm for (d).

512, posterior pituitary homogenates were fractionated by centrifugation on sucrose density gradients. Sucrose density gradients separate intracellular particles based on their densities and have been used extensively to study the association of membrane proteins with neurosecretory granules and synaptic vesicles (Obendorf *et al.*, 1988; Navone *et al.*, 1989; Walch-Solimena *et al.*, 1993; Clift-O'Grady *et al.*, 1990). As shown in Figure 7, the peak of the 70 kDa protein recognized by anti-ICA 512 antibodies was recovered in dense fractions (sucrose molarity = 1.75) and co-localized with the peak of the secretory granule marker secretogranin II (Rosa *et al.*, 1985). A second peak of ICA 512 was detected in a lighter fraction (fraction 11; sucrose molarity = 1.14), and overlapped with Rab5, a small GTP binding protein associated with endosomes (Chavrier *et al.*, 1990). The synaptic vesicle marker synaptophysin (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Buckley *et al.*, 1987) migrated at a lower density, consistent with our electron microscopy findings, indicating that ICA 512 is not associated with synaptic vesicles. These data demonstrated that the 70 kDa protein recognized by anti-ICA 512 antibodies is concentrated primarily on secretory granules, whereas a smaller pool of the protein partially co-distributes with Rab5, possibly reflecting its recycling to an early endosomal compartment following retrieval from the cell surface.

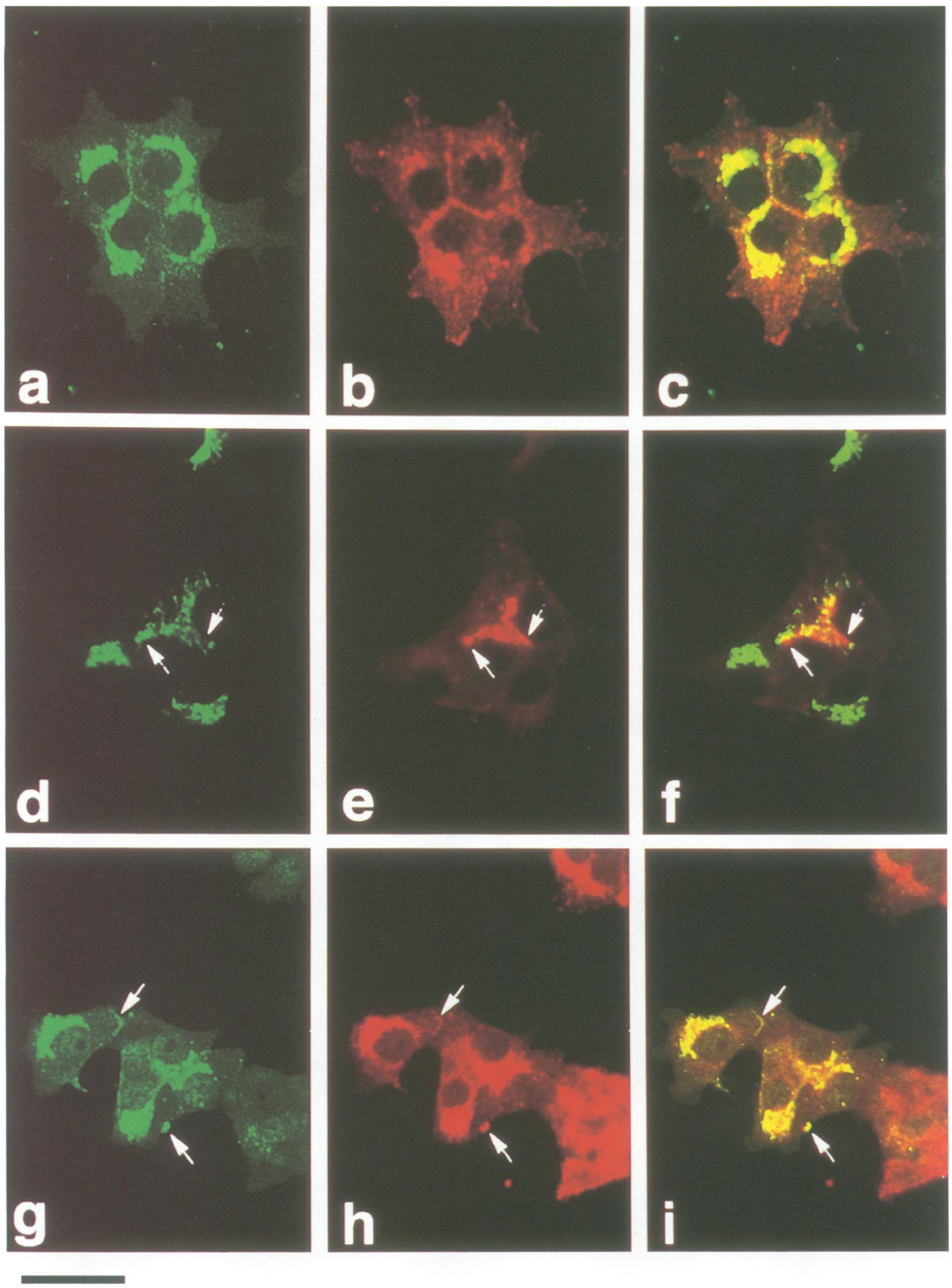

Fig. 5. Uptake of anti-ICA 512 luminal domain antibodies in RIN cells as visualized by confocal microscopy. After incubation for 1 h with 92-18 serum in stimulating media (55 mM KCl), cells were either immediately fixed and permeabilized (**a–c**) or allowed to recover for 14 h before immunostaining (**d–i**). Cells were double immunolabeled with anti-transferrin receptor (a–f) or anti-insulin antibodies (g–i) (green pseudocolor). (a–c) Uptaken anti-ICA 512 ectodomain antibodies (red pseudocolor) are detectable in the proximity of the plasma membrane as well as in the perinuclear region and throughout the cytoplasm. Note the partial co-localization with transferrin receptor (d–i). After 14 h recovery, anti-ICA 512 ectodomain antibodies co-localize extensively with insulin both in the Golgi complex region and at the cell periphery (g–i, arrows). On the other hand, internalized anti-ICA 512 ectodomain antibodies and transferrin receptor very often have a complementary distribution (d–f, arrows). Scale bar in (a) is 60 μm for (a–c) and 70 μm for (d–i) .

The 70 kDa protein recognized by anti-ICA 512 antibodies corresponds to the transmembrane fragment of ICA 512

To establish the relationship between ICA 512 and the 70 kDa protein detected by Western blot with anti-ICA 512 antibodies, the 70 kDa protein was affinity purified from bovine posterior pituitary homogenates using the antiserum directed against the ICA 512 cytoplasmic domain and processed for NH_2-terminal microsequencing. This microsequence, which includes 14 amino acids, was 79% identical and 93% similar to the predicted sequence of human ICA 512 starting from its serine residue at position 449 (Figure 8). In human and mouse ICA 512, Ser449 is immediately preceded by two lysine residues at positions 447 and 448. Since cleavage by endoproteases after two consecutive basic amino acids is a common mechanism by which proproteins of the secretory granules are converted to their mature form, this result strongly

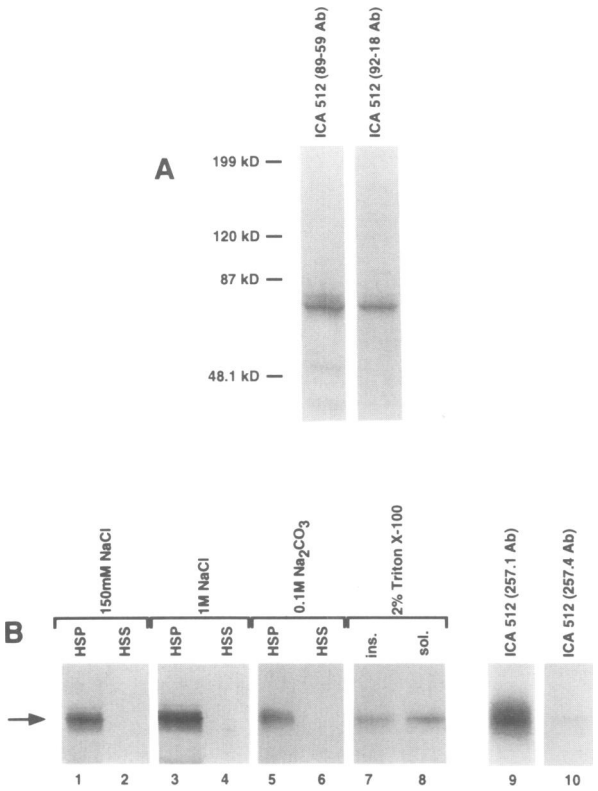

Fig. 6. (**A**) Western blotting on bovine posterior pituitary post-nuclear supernatants. Thirty μg of protein were separated on 10% SDS–polyacrylamide gel and Western blotted as follows: 89-59 rabbit serum (lane 1); 92-18 rabbit serum (lane 2). Immunoreactivity was detected with alkaline phophatase-conjugated goat anti-rabbit antibodies. A broad protein of ~70 kDa is the only protein recognized by both 89-59 and 92-18 anti-ICA 512 antibodies. (**B**) Western blotting with 89-59 anti-ICA 512 antiserum on bovine posterior pituitary subcellular fractions (lanes 1–8) and immunoprecipitates (IPP) with anti-ICA 512 257.1 and 257.4 monoclonal antibodies (lanes 9 and 10). HSP = high speed pellet; HSS = high speed supernatant; ins. = 2% Triton X-100-insoluble material; sol. = 2% Triton X-100-soluble material. ICA 512 is only detected in HSP (lanes 1–2), even after treatments with 1 M NaCl (lanes 3–4) or 0.1 Na_2CO_3, pH 11.5 (lanes 5–6). Following extraction with 2% Triton X-100, most ICA 512 is recovered in the detergent-soluble material (lane 8), although a considerable amount of the protein is present in the insoluble material (lane 7). Both anti-ICA 512 monoclonal antibodies immunoprecipitated the 70 kDa protein recognized by the 89-59 anti-ICA 512 antiserum (lanes 10 and 11).

suggests that a proteolytic cleavage between amino acids 448 and 449 is responsible for the generation of the 70 kDa ICA 512 transmembrane fragment. Based on the human and mouse ICA 512 sequence, this transmembrane fragment has a predicted mol. wt of 59 kDa (aa 449–979). The presence in this fragment of the two putative *N*-glycosylation sites of ICA 512 could easily account for a shift in its electrophoretic mobility to 70 kDa. Preliminary evidence suggests that ICA 512 is indeed *N*-glycosylated (R.Dirkx, D.Rabin and M.Solimena, unpublished observations). Furthermore, the 70 kDa protein recognized by both anti-ICA 512 antisera has an isoelectric point of 6.0 (Figure 9), a value which corresponds to the predicted isoelectric point of the ICA 512 transmembrane fragment generated after cleavage between amino acids 448 and 449. The predicted isoelectric point of full-length human/mouse ICA 512, on the other hand, is 6.7. These data conclusively demonstrated that the 70 kDa protein recognized by anti-ICA 512 antibodies corresponds to the transmembrane fragment of ICA 512.

Presence of anti-ICA 512 autoantibodies in the large majority of IDDM patients

Anti-ICA 512 autoantibodies were detected previously by enzyme-linked immunosorbent assay (ELISA) in 48% of newly diagnosed IDDM patients using as substrate antigen a recombinant ICA 512 fragment including amino acids 643–979 (Rabin *et al.*, 1994). To assess whether the native protein could yield a high diagnostic sensitivity, we measured the prevalence of anti-ICA 512 autoantibodies in human sera by immunoprecipitation of native ICA 512 from bovine posterior pituitary Triton X-100 extracts. Fifteen out of 17 (88%) sera from newly diagnosed IDDM patients plus the serum from a pre-diabetic subject, but none of 10 sera from healthy control subjects, immunoprecipitated the 70 kDa form of ICA 512 (Figure 10). By comparison, only 5/17 (29%) IDDM sera plus the pre-diabetic serum were found positive for anti-ICA autoantibodies when tested by ELISA with the recombinant ICA 512 fragment. These results suggest that anti-ICA 512 autoantibodies are present in the vast majority of newly-diagnosed IDDM patients, can precede the onset of the disease, and could be as common a humoral marker of IDDM as anti-GAD autoantibodies.

Discussion

The results presented here indicate that the autoantigen of IDDM ICA 512 is an intrinsic membrane protein of peptide-containing secretory granules of endocrine cells and neurons. ICA 512 joins a growing list of transmembrane proteins of secretory granules, including vacuolar type proton-pumping ATPase (Nelson and Lill, 1994), cytochrome b_{561} (Silsand and Flatmark, 1974), nucleotide carrier (Kostron *et al.*, 1977), vesicular amine transporter (VAT) (Erickson *et al.*, 1992), SV2 (Lowe *et al.*, 1988; Feany *et al.*, 1992) peptidyl α-amidating monooxygenase (PAM) (Eipper *et al.*, 1993), glycoprotein II (Obendorf *et al.*, 1988), synaptophysin (Lowe *et al.*, 1988), synaptotagmin (Lowe *et al.*, 1988; Walch-Solimena *et al.*, 1993), synaptobrevins (Chilcote *et al.*, 1995), secretory carrier membrane proteins (SCAMPs) (Brand *et al.*, 1991, Brand and Castle, 1993) and secretory granule-specific antigen (SGSA) (Yamashita and Yasuda, 1992).

Several features, however, distinguish ICA 512 from other constituents of secretory granule membranes. Because of its single transmembrane domain and its structural homology with receptor-type PTPases, ICA 512, unlike V_0-ATPase, cytochrome b_{561}, nucleotide carrier, VAT, SV2 and PAM, is probably involved in mechanisms other than transporting molecules across the granule membrane or processing secretory content proteins. Unlike glycoprotein II and SGSA, there is no evidence suggesting the presence of ICA 512 on lysosomes or secretory granules of exocrine cells. Synaptophysin, synaptotagmin and synaptobrevin are present on secretory granules, but appear to be mostly enriched on synaptic vesicles. While we cannot totally rule out the possibility that a minor pool of ICA 512 may be associated with synaptic vesicles, our morphological and biochemical data indicate that the protein is associated primarily with neurosecretory

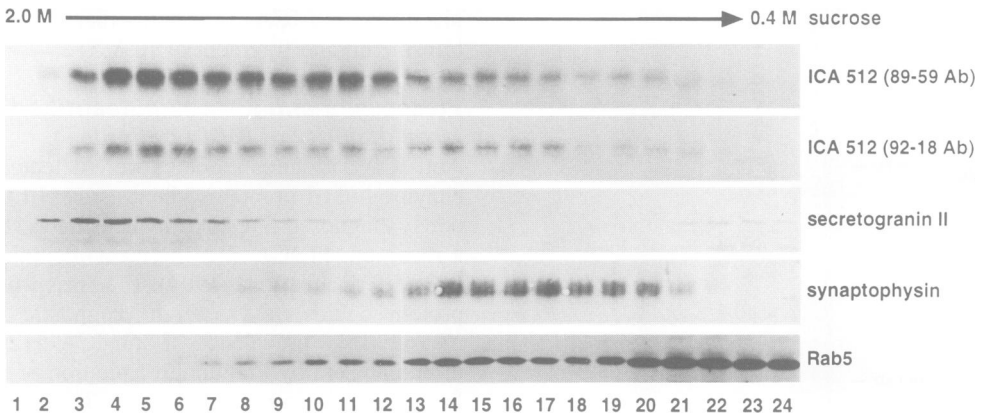

Fig. 7. Western blotting of continuous sucrose gradient fractions (0.4–2.0 M sucrose) of bovine posterior pituitary. Equal volumes of each fraction were separated on 10% SDS–polyacrylamide gels and Western blotted with rabbit antisera directed against ICA 512, the secretory granule marker secretogranin II, the synaptic vesicle marker synaptophysin and the early endosomal marker Rab5. The peak of ICA 512 is found in dense sucrose fractions (1.75 M sucrose) and overlaps with the peak for secretogranin II, whereas synaptophysin peaks in 0.8–1.0 M sucrose fractions. A smaller peak of ICA 512 co-localizes with Rab5 (fraction 11, 1.14 M sucrose). Lysis of some secretory granules during the fractionation procedure explains the recovery of a pool of secretogranin II, a content protein of secretory granules, in light sucrose fractions. Unlike secretogranin II, the 70 kDa protein recognized by anti-ICA 512 antibodies is not present in light sucrose fractions, as expected for an intrisic membrane protein. The presence of a large pool of Rab5 in light sucrose fractions as well as in synaptophysin-enriched fractions is consistent with Rab5 being a cytosolic protein partially associated with synaptic vesicles (Fischer von Mollard *et al.*, 1994).

granules. In view of these considerations, ICA 512 is likely to be involved in processes specific for neurosecretory granules, such as their biogenesis, trafficking or regulated exocytosis. Furthermore, its virtually ubiquitous expression in neuroendocrine cells suggests that ICA 512 has a general role in neuroendocrine functions.

According to the primary amino acid sequence, the mol. wt of ICA 512 is 106 kDa (Lan *et al.*, 1994). This is considerably larger than the protein of 70 kDa recognized by both rabbit antibodies directed against distinct domains of ICA 512. Several independent pieces of evidence, including tissue distribution, subcellular localization, immunological cross-reactivity, biochemical properties and sequence homology, indicate that the 70 kDa protein derives from the proteolytic cleavage of ICA 512 between residues 448 and 449. Cleavage after lysines 447–448 is in agreement with the dibasic consensus sequence recognized by many convertases, although cleavage at monobasic residues has also been described (Seidah *et al.*, 1994). Sucrose density gradients of bovine posterior pituitary homogenates demonstrated that secretory granules contain the 70 kDa form of the protein. Moreover, we have never detected an ICA 512 form larger than 70 kDa in this tissue. Thus, protein conversion must occur somewhere between the endoplasmic reticulum and mature secretory granules. Probable locations for this conversion are the *trans*-Golgi network or immature secretory granules, the compartments where proteolytic cleavage of secretory granule proteins frequently takes place (Orci *et al.*, 1987). Processing of ICA 512 at its luminal domain resembles that of other members of the receptor-type PTPase family, including LAR (Streuli *et al.*, 1992; Serra-Pages *et al.*, 1994), PTPκ (Jiang *et al.*, 1993) and PTPμ (Brady-Kalnay and Tonks, 1994). In these PTPases, proteolytic cleavage of the proprotein results in the generation of two subunits which remain associated in a heterodimeric complex. For ICA 512, it is still unclear whether the two ICA 512 fragments generated by cleavage between amino acids 448 and 449 remain associated in a heterodimeric

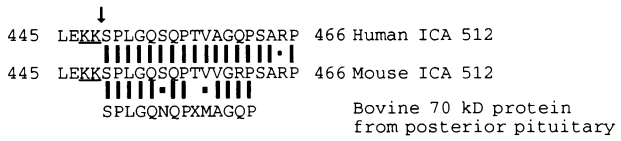

Fig. 8. Alignment of the human and mouse ICA 512 sequences with the new NH_2-terminal microsequence of the 70 kDa protein purified from bovine posterior pituitary. The two lysines preceding the cleavage site (arrow) are underlined.

complex. It is possible that the ectodomain of ICA 512 may undergo multiple proteolytic cleavages as often described for proteins sorted in secretory granules and for LAR (Serra-Pages *et al.*, 1994). Analysis of available ICA 512 sequences indicates that, in addition to the known processing site between residues 448 and 449, its luminal domain contains at least three other conserved dibasic sites which are potential cleavage sites. The fragments of ICA 512 resulting from these putative cuts may be secreted and have biological activity, either as signaling molecules or by interacting with extracellular matrix molecules.

To our knowledge, ICA 512 is the first member of the receptor-type PTPase family found to be resident in an intracellular compartment, although its function is still unknown. Previous experiments failed to detect PTPase activity of recombinant ICA 512 expressed in bacteria when tested with several common PTPase substrates. These data suggested either that ICA 512 has a very narrow substrate specificity or that ICA 512 is not a conventional PTPase. In particular, it was noticed that the PTPase domain of ICA 512 contains an aspartate instead of an alanine at position 911, a substitution known to abolish PTPase activity (Zhang *et al.*, 1994). Our uptake studies with anti-ICA 512 ectodomain antibodies show that, upon exposure at the cell surface, ICA 512 recycles to the region of the Golgi complex and it is sorted into newly formed secretory granules, thus implying its participation in several rounds of exo–endocytosis.

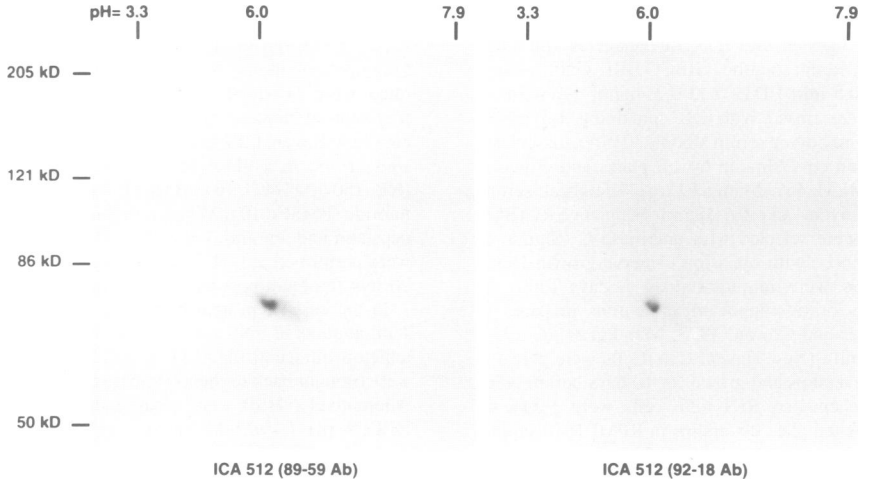

Fig. 9. Western blotting for ICA 512 following two-dimensional gel electrophoresis of bovine posterior pituitary high speed pellets. 89-59 and 92-18 anti-ICA 512 antibodies both recognize a single protein spot of ~70 kDa with an isoelectric point of 6.0.

Interestingly, we observed that, after Triton X-100 extraction of bovine posterior pituitary, a significant pool of the 70 kDa transmembrane fragment of ICA 512 was recovered in the detergent-insoluble pellet, hinting at a stable interaction of a pool of the protein with the cytomatrix surrounding secretory granules.

The demonstration that ICA 512 is an intrinsic membrane protein of secretory granules supports the close correlation which exists between autoimmunity associated with β-cell secretory vesicles and IDDM. Among the various protein antigens of IDDM thus far characterized, ICA 512 presents unique features. No other IDDM autoantigen is an intrinsic membrane protein. They are either cytosolic proteins associated with secretory vesicles, such as GAD, or content proteins of the secretory granules such as insulin and carboxypeptidase H. Thus, it is likely that circulating autoantibodies against these antigens cannot affect β-cells directly. Conversely, the presence of ICA 512 on secretory granules leads to the question of whether anti-ICA 512 autoantibodies directed against its luminal domain may impair the normal activity of β-cells. As anti-ICA 512 luminal antibodies are taken up by RIN cells, patient autoantibodies directed against the luminal domain of ICA 512 could be internalized by β-cells and affect insulin secretion. Our data indicate that anti-ICA 512 antibodies are present in 88% of newly diagnosed IDDM patients and can precede the onset of the disease. Thus, regardless of their possible contribution to the pathogenesis of IDDM, anti-ICA 512 autoantibodies appear to be a sensitive marker for the diagnosis of the disease. Future studies will be required to assess how frequently anti-ICA 512 antibodies precede the onset of diabetes, their epitope specificity and their predictive value for IDDM.

Materials and methods

Antibodies

Two antisera directed against the ICA 512 cytoplasmic (89-59) and luminal (92-18) domains were generated by immunizing rabbits with the recombinant ICA 512 fragments encompassing amino acids 643–979 and 389–576, respectively. Anti-ICA 512 monoclonal antibodies 257.1 and 257.4 were generated by immunizing mice with the glutathione *S*-transferase (GST)–ICA 512 fragment including amino acids 643–979.

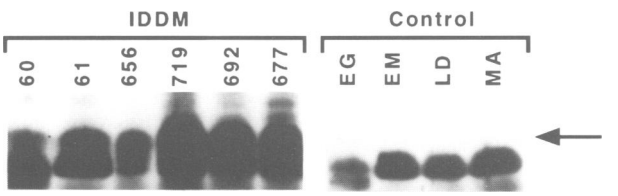

Fig. 10. Western blotting with 89-59 anti-ICA 512 serum on immunoprecipitates from Triton X-100 extracts of bovine posterior pituitary high speed pellets with human sera. Subject sera are identified with their number or letter codes. Serum 61 was from a pre-diabetic subject positive for islet cell autoantibodies. The 70 kDa form of ICA 512 (arrow) is immunoprecipitated by the sera from diabetic and pre-diabetic patients, but not by sera from control subjects.

To assess the specific reactivity of 89-59 and 92-18 sera with ICA 512, 4 μl of each sera were incubated on ice for 30 min with 1.4 μM ICA 512 recombinant immunizing fragments in 1 ml of blotto solution (5% non-fat dry milk, 10 mM Tris–HCl, pH 7.4, 0.1% Tween 20) prior to Western blotting on 30 μg of protein from bovine posterior pituitary post-nuclear supernatants. Anti-ICA 512 89-59 and 92-18 antibodies were affinity purified on ICA 512 recombinant peptides 643–979 or 389–576 which had been cleaved and separated from GST. These peptides were coupled to agarose beads using an AminoLink kit (Pierce, Rockford, IL). Antibodies were eluted from the columns with 0.1 M acetic acid, pH 3.0 and immediately brought back to neutral pH with 1 M Tris pH 8.0.

A mouse monoclonal antibody directed against GAD65, MN65, was generated by immunizing mice with a synthetic peptide corresponding to amino acids 4–21 of GAD65 (R.Dirkx, M.Radzynski and M.Solimena, unpublished results). Characterization of this antibody will be described elsewhere. The following antibodies were generous gifts: mouse monoclonal antibodies directed against synapsin and synaptophysin as well as rabbit serum directed against synaptophysin (Dr R.Jahn, New Haven, CT); rabbit serum directed against secretogranin II (Dr P.Rosa, Milano, Italy); mouse monoclonal antibody directed against lgp 120 and rabbit serum directed against Rab5 (Dr I.Mellman, New Haven, CT); and mouse monoclonal antibody directed against transferrin receptor (Dr I.S.Trowbridge, San Diego, CA). The following antibodies were from commercial sources: anti-glucagon and anti-somatostatin mouse monoclonal antibodies (Sigma); chicken anti-human insulin IgG and affinity-purified mouse anti-rabbit IgG (Accurate, Westbury, NY); anti-MAP2 mouse monoclonal antibody, goat anti-rabbit and goat anti-mouse IgG conjugated to fluorescein or lissamine rhodamine (Boeringher Mannheim, Indianapolis, IN); goat anti-rabbit and goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA); and goat anti-rabbit IgG conjugated to 10 nm gold.

Cell cultures

Primary hypothalamic cell cultures were prepared from the hypothalamus of 1–2-day-old rats. Hypothalami were dissected under oxygenated DB

buffer (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 0.4 mM $CaCl_2$, 20 mM HEPES, pH 7.2) and digested with 7.5 U/ml papain (Worthington, Freehold, NJ) in Earl's balanced salt solution (GIBCO BRL, Gaithersburg, MD) with 1.5 mM $CaCl_2$, 0.5 mM EDTA and 0.2 mg/ml L-cysteine at 37°C for 20 min. Cells were rinsed with DB containing 1.5 mg/ml trypsin inhibitor and 1.5 mg/ml bovine serum albumin (BSA), dissociated with Pasteur pipets, plated on coverslips in 6-well plates and grown in medium containing 45% DMEM, 45% Mam's F12, 10% fetal calf serum, 10 μg/ml penicillin/streptomycin and 0.5 μg/ml Fungizone (GIBCO BRL). Non-neuronal cells were selected with arabinose C (Sigma) as described (Ronnett *et al.*, 1991), with omission of nerve growth factor. Hypothalamic cultured cells were immunostained 5 days following dissection. Primary cultures of rat hippocampal neurons prepared as previously described (Banker and Cowan, 1977; Mundigl *et al.*, 1993) were donated by Dr De Camilli (New Haven, CT). Cells were plated at a density of 4000/cm^2 on coverslips and grown for 10 days before being processed for immunocytochemistry. RIN m5F cells were grown on polyornithine- (10 μg/ml) coated glass coverslips in RPMI 1640 supplemented with 10% serum and incubated in 7.5% CO_2.

Immunofluorescence

For immunofluorescence *in situ*, rat tissues were fixed by transcardiac perfusion with 4% paraformaldehyde in 120 mM sodium phosphate buffer. Single and double immunolabeling on 12 μm cryostat tissue sections was performed as previously described (De Camilli *et al.*, 1983). RIN m5F (50% confluency) and cultured neuronal cells were immunostained as described (Reetz *et al.*, 1991; Mundigl *et al.*, 1993). Dilutions of primary antibodies were as follows: 92-18 pre-immune serum, anti-ICA 512 89-59 and 92-18 sera, mouse anti-synaptophysin and anti-MAP2 antibodies, 1:50; anti-synapsin and anti-insulin antibodies, 1:100; anti-somatostatin and anti-glucagon antibodies, 1:1000; anti-lgp 120 hybridoma supernatant, 1:10; anti-GAD65 MN65 hybridoma supernatant, 1:2; and anti-transferrin receptor ascites 1:50. Secondary antibodies conjugated to rhodamine or fluorescein were used at 1:50 or 1:30 dilution, respectively. Slides were analyzed with an Olympus B-Max 40 microscope equipped with epifluorescence, and photomicrographs were taken with Kodak T-Max 100 film (Rochester, NY). Confocal microscopy was performed using a Bio-Rad MRC-600 system equipped with a krypton–argon laser attached to a Zeiss Axiovert microscope (Zeiss, Thornwood, NY). Acquired images were processed with Adobe Photoshop software (Adobe System Inc., Mountain View, CA) as described previously (Xu *et al.*, 1992).

Uptake of anti-ICA 512 antibodies in RIN cells

RIN cells m5F grown on glass coverslips (50% confluency) were incubated at 37°C for 2 min, 10 min or 1 h in 1 ml Krebs–Ringer HEPES (KRH) buffer containing 1 mM or 55 mM KCl and one of the following rabbit sera: 92-18 pre-immune serum, anti-ICA 512 92-18 or 89-59 serum at a dilution of 1:50. The composition of 1 mM KCl KRH was 130 mM NaCl, 1 mM KCl, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$, 20 mM HEPES, pH 7.3 and 11 mM glucose. The composition of 55 mM KCl KRH was 76 mM NaCl, 55 mM KCl, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$, 20 mM HEPES, pH 7.3 and 11 mM glucose. After removal of the antibodies, the cells were washed once with the respective KRH buffer and then were either fixed with 4% paraformaldehyde or incubated in culture media for an additional 14 h before fixation. Immunoglobulin uptake was visualized with rhodamine-conjugated goat anti-rabbit IgG in goat serum dilution buffer (16% filtered goat serum, 0.45 M NaCl, 0.3% Triton X-100, 20 mM $NaPO_4$ buffer, pH 7.4). Double immunolabelings and confocal microscopy were performed as described above.

Immunoelectron microscopy

Immunocytochemistry on cryosections was performed as previously described (Webster *et al.*, 1994). Briefly, small pieces of rat posterior pituitary fixed with 4% paraformaldehyde were embedded in 10% gelatin, infiltrated with 2.3 M sucrose and polyvinylpyrrolidone, and sectioned with a diamond knife at temperatures between −110 and −130°C in an Ultracut E cryomicrotome (Leica, Deerfield, IL) cooled with liquid nitrogen. Ultrathin sections (~100 nm) were mounted on copper- or nickel-coated specimen grids and immunolabeled with primary antibodies (92-18 pre-immune serum, anti-ICA 512 89-59 and 92-18 and anti-synaptophysin sera) at 1:10 dilution followed by goat anti-rabbit IgG conjugated to 10 nm gold particles at 1:10 dilution. Immunolabeled sections were examined with a Phillips CM 10 electron microscope.

Subcellular fractionation

Fresh bovine posterior pituitaries were collected at a local abattoir. Fragments of mouse insulinoma from NOD-scid/RIP-SV40 transgenic mice were provided by Dr D.Serreze (Bar Harbor, ME). Surgical fragments of human pituitary adenoma were provided by Dr A.deLotbiniere (New Haven, CT). Bovine, mouse and human tissues were homogenized on ice in a glass Teflon homogenizer in homogenization buffer [HB, 150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine and 1 μg/ml of leupeptin, antipain, pepstatin and aprotinin] at a 1:10 w/v ratio. All biochemical procedures were performed at 0–4°C. Crude homogenates were spun at 1000 *g* for 20 min to obtain post-nuclear supernatants (PNS).

In the case of protein homogenates from bovine posterior pituitary, 1 ml aliquots of PNS were spun at 100 000 *g* for 30 min in a Beckman tabletop ultracentrifuge (TLA 100.2 rotor). High speed pellets (HSP) were brought back to their original volumes with homogenization buffer. Alternatively, HSP were resuspended in either 1 M NaCl, 10 mM HEPES, pH 7.4; 0.1 M Na_2CO_3, pH 11.5 or in homogenization buffer containing 2% Triton X-100. Resuspended pellets were incubated on ice for 30 min and spun at 100 000 *g* as described above. The resulting HSP were once again brought back to their original volumes. Equal volumes of the high speed supernatants (HSS) and HSP were separated on 10% SDS–polyacrylamide gels. Western blotting was performed with 89-59 or 92-18 sera at 1: 250 dilution followed by alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:6000 dilution.

Fractionation on sucrose density gradients

Fractionation of bovine neurosecretosomes on continuous sucrose density gradients (from 2.0 M to 0.4 M sucrose) was performed as previously described (Walch-Solimena *et al.*, 1994). For these experiments, fresh bovine posterior pituitaries were homogenized in 0.32 M sucrose, 4 mM HEPES, pH 7.4, 2 μg/ml pepstatin and 0.1 mM PMSF. For Western blotting, rabbit sera directed against ICA 512, secretogranin II, synaptophysin or Rab 5 were all diluted 1:1000 followed by alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:6000 dilution.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to O'Farrell (1975) with slight modifications. Briefly, 500 μg of bovine posterior pituitary HSP protein were isoelectrofocused with 2% ampholines pH 5–7 and 0.8% ampholines pH 3–10 (Bio-Rad) at 100–400 V for 2 h, 400 V for the next 16 h and 800 V for the last 30 min. The second dimension was performed on 8% SDS–polyacrylamide gels. Western blottings were performed using 89-59 serum and 92-18 affinity-purified antibodies at 1:100 and 1:50 dilutions, respectively, followed by peroxidase-conjugated goat anti-rabbit IgG at 1:1000 dilution. Immunoreactivity was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL), according to the manufacturer's instructions. Protein concentration was determined with the BCA method (Pierce).

ICA 512 affinity purification and microsequencing

An antibody chromatography column was prepared by coupling anti-ICA 512 89–59 affinity-purified antibodies to agarose beads with the AminoLink kit (Pierce). Bovine posterior pituitary HSP protein (20 mg) was solubilized in 2% Triton X-100 with proteases inhibitors, loaded on the resin and washed with 2% Triton X-100 in phosphate-buffered saline (PBS) and then with PBS. Protein was eluted with 2% SDS–gel electrophoresis sample buffer, separated on a 10–20% tricine gel (Novex, San Diego, CA) blotted on a PVDF membrane (Bio-Rad) and stained with Coomassie Blue. The 70 kDa protein (12.7 pmol) purified with anti-ICA 512 antibodies was processed for NH_2-terminal sequencing by Edman degradation using an Applied Biosystem model 477A Protein Sequencer operated in the gas phase with an on-line model 120A Analyzer and Turbochrome 4.0 Chromatography software.

Detection of anti-ICA 512 autoantibodies in human sera

The sera of 17 newly diagnosed IDDM patients and one of a pre-diabetic patient positive for islet cell autoantibodies were provided by Å.Lernmark (Seattle, WA), and by U.Di Mario and C.Tiberti (Rome, Italy). Control sera from 10 healthy subjects were collected at the Center for Clinical Research at Yale University School of Medicine. For immunoprecipitation experiments, 2% Triton X-100-soluble extracts of 200 μg of protein from bovine posterior pituitary HSP resuspended in 1 ml of HB were incubated for 12 h with 25 μl of human sera at 4°C, followed by 100 μl of 50% protein G–Sepharose beads (Pharmacia, Piscataway, NJ) for 1 h at 4°C. Bead pellets were washed three times with HB

and solubilized with Laemmli sample buffer. Immunoprecipitates were separated on 8% SDS–polyacrylamide gels and immunoblotted with 89-59 serum at 1:100 dilution, followed by mouse anti-rabbit affinity-purified IgG at 1:1000 dilution and alkaline phosphatase-conjugated goat anti-mouse IgG at 1:5000 dilution. ELISA with the ICA 512 recombinant fragment 643–979 was performed as described (Rabin *et al.*, 1994).

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