MECHANISMS OF ACTION OF ISLET NEOGENESIS ASSOCIATED PROTEIN 
(INGAP): COMPARISON OF THE FULL-LENGTH RECOMBINANT PROTEIN AND 
A BIO-ACTIVE PEPTIDE

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Abbreviated title: Mechanisms of action of INGAP- protein and INGAP-peptide

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Abstract

Islet Neogenesis Associated Protein (INGAP) was discovered in the partially duct-obstructed hamster pancreas, as a factor inducing formation of new duct-associated islets. A bio-active portion of INGAP, INGAP\textsuperscript{104-118} peptide (INGAP-P), has been shown to have neogenic and insulin-potentiating activity in numerous studies including recent Phase 2 clinical trials that demonstrated improved glucose homeostasis in both Type1 and Type2 diabetic patients. Aiming to improve INGAP-P efficacy and to understand its mechanism of action, we cloned the full-length protein (rINGAP) and compared the signaling events induced by the protein and the peptide in RIN-m5F cells that respond to INGAP with an increase in proliferation. Here we show that although both rINGAP and INGAP-P signal via the Ras-Raf-Erk pathway, rINGAP is at least a hundred times more efficient on a molar basis than INGAP-P. For either ligand, Erk1/2 activation appears to be pertussis toxin-sensitive, suggesting involvement of a G-protein coupled receptor(s). However, there are clear differences between the peptide and the protein in interactions with the cell surface and in the downstream signaling. We demonstrate that fluorescent-labeled rINGAP is characterized by clustering on the membrane and by slow internalization (up to 5h), whereas INGAP-P does not cluster and is internalized within minutes. Signaling by rINGAP appears to involve Src, in contrast to INGAP-P, which appears to activate Akt in addition to the Ras-Raf-Erk1/2 pathway. Our data thus suggest that interactions of INGAP with the cell surface are important to consider for further development of INGAP as a pharmacotherapy for diabetes.

Key words: islet neogenesis, INGAP, Reg proteins, RIN-m5F cells, proliferation, signalling
**Introduction**

Regeneration of β-cells in diabetic patients is an important goal of diabetes research. In recent years, there has been increasing interest in the development of new strategies to induce β-cell regeneration and new islet formation *in situ* (3, 28, 40). Identification of bioactive molecules with the capacity to stimulate expansion of the remaining β-cell mass or with islet neogenic activity is therefore crucial for harnessing the regenerative potential of the native pancreas.

Islet Neogenesis Associated Protein (INGAP) is a 16.8 kDa protein originally identified in a crude extract from a partially obstructed hamster pancreas (42). INGAP is expressed in the pancreas and duodenum (22, 39, 41) and has been shown to induce islet neogenesis in several species (43, 44). Structurally, INGAP is a member of the Reg family of secreted C-type lectins that comprises more than 25 members, classified into 4 subfamilies based on the primary sequence (33, 58). INGAP belongs to the large Reg3 subfamily that has been identified predominantly in gastrointestinal tissues (pancreas, stomach, liver) in rat, mouse, hamster and humans (20, 39, 53, 56). Despite the ubiquity of Reg proteins, not much is known about their functions and the mechanisms of action. While there is a consensus on the role of Reg1 as a β-cell mitogen (33, 52, 53, 56), much less is known about the functions of the Reg3 family. Expression of several Reg proteins, including INGAP, is influenced by a number of inflammatory cytokines (1, 19, 20, 36) or bacterial infection (32), thus implicating these proteins in the acute phase response. Consistent with being C-type lectins, two members of the Reg3 family, α and γ, have been shown to bind carbohydrate ligands, such as mannan and peptidoglycans, and to have direct antimicrobial effects (10, 11). Human Reg3α has also been shown to bind lactose and extra-cellular matrix (ECM) proteins and to induce adhesion of
hepatocytes *in vitro* (12, 13). While it remains to be determined if other members of the family are able to act similarly, a number of studies suggest that Regs may bind specific cell-surface receptors, (perhaps in addition to, or as an alternative to binding carbohydrates) and activate multiple signaling pathways (6, 23, 26, 50). To date, the only known Reg receptor (EXTL3), which specifically binds Reg1, is a transmembrane glycosyltransferase homologous to the multiple exostoses-like gene family (26). It is not known whether this receptor binds INGAP and other Reg proteins, or whether a different receptor (if any) is involved in the mechanism of INGAP action.

One argument in favor of the receptor hypothesis is that the biological activity of INGAP appears to be mediated by a 15 amino acid fragment of the protein (aa 104-118), namely INGAP peptide (INGAP-P), which consists of a highly conserved IGLHDP motif and a unique sequence SHGTLPNGS not found in the other members of the Reg family (39). Synthetic INGAP peptide has been demonstrated to be as effective as the protein in inducing new islet formation and reversing streptozotocin-induced diabetes in hamsters and mice (43, 44) and is, therefore, a possible ligand for the receptor. Biological effects of a synthetic INGAP-P have been extensively studied both *in vitro* and *in vivo* in our laboratory and by others. To date, the most compelling data show that INGAP-P: 1) induces *in vitro* regeneration of functional human islets from dedifferentiated, islet-derived duct-like structures (25); 2) dose dependently stimulates expansion of β-cell mass in rodents, dogs and cynomolgus monkeys (29, 38, 43); and 3) increases insulin secretion and β-cell size and upregulates the expression of several genes related to β-cell function in rat neonatal islets *in vitro* (4, 5, 7). These important results were followed by clinical trials to investigate its efficacy and safety in humans, in which INGAP-P was found to have a signal effect with an improvement of glucose homeostasis confirmed by A1C reduction at 90
days in patients with Type 2 diabetes and by a significant increase in C-peptide secretion in 
patients with Type 1 diabetes (18, 54).

Taken together, these data support the potential of INGAP as a candidate agent for the 
treatment of diabetes that possesses both islet-neogenic and insulinotropic activities. However, 
the relatively short plasma half-life of INGAP-P and the need for administration in a high dose 
calls for improvement of the drug profile (18). In this context, characterization of the mechanism 
of action becomes an important priority for further research.

For this reason, we have recently cloned full length recombinant protein (rINGAP) which is 
much more stable than the peptide (at least 5 days in cell culture) and is 6His-tagged (2). To 
provide a detailed, side-by-side comparison of biological effects of INGAP-P and the full-length 
protein, we chose a well characterized β-cell line, RIN-m5F (14), that responds to INGAP-P by 
an increase in proliferation (34). Our data show that rINGAP is a hundred times more potent on a 
molar basis than INGAP-P and that, although they both signal via activation of Ras-Raf-Erk 
pathway, the upstream signaling events may differ between the protein and peptide. Using 
confocal microscopy, we found several differences between the protein and peptide in cell 
binding and internalization. We show that fluorescent-labeled rINGAP forms patches on the cell 
surface in a fashion consistent with receptor binding and clustering, whereas INGAP-P rapidly 
internalizes. INGAP-induced activation of Erk1/2 is significantly reduced by pertussis toxin (for 
both protein and peptide) thus suggesting that despite differences in cell binding, both INGAPs 
act via a G-protein coupled receptor.
Recombinant INGAP and INGAP-peptide - INGAP peptide (INGAP-P), a 15-amino acid fragment of INGAP protein (aa 104-118; MW 1501.6) was synthesized and HPLC-purified at the Sheldon Biotechnology Centre (McGill University, Montreal). A full-length recombinant INGAP (rINGAP) containing C-terminal 6-His tag (MW 17.6 kDa) was cloned from hamster pancreatic tissue by directional cloning of the PCR product generated with Superscript III RT and Platinum™ Pfx DNA Polymerase (Invitrogen) into pcDNA3.1D/V5-His-TOPO™ expression vector (Invitrogen). This construct was used for re-cloning into a lentiviral vector and expression in H293 cells (described in detail in (2)). Purification of rINGAP was carried out using Cobalt resin (BD TALON™, BD Biosciences, or Fractogel EMD Chelate(M), Merck) as described (2).

Cell culture - RIN-m5F cells (passage 18) were purchased from ATCC and maintained at 37°C/ 5% CO₂ in RPMI-1640 medium (Invitrogen) containing 25mM glucose, 10% FBS (Montreal Biotech), and antibiotics/antimycotics (Invitrogen). The following experiments were carried out on cells from passages 25-31. Cells were plated in 60mm TC dishes (1x10⁶ cells per dish) and allowed to grow for 24-48h, followed by the serum withdrawal for 24h prior to the treatment. INGAP-P, rINGAP, EGF (10ng/ml, Sigma) or Exendin 4 (Ex4, 10nM, Bachem) were administered in serum-free medium for the times indicated. For the analysis of signaling pathways, cells were pretreated for 40 min with the following inhibitors (all from Calbiochem, concentrations were calculated to exceed IC₅₀ 10 to 20 times, or as suggested by the manufacturer): 100nM Wortmannin, (abbreviated as Wm, PI3K pathway), 10μM PD98059 (PD98, MEK), 100nM AG1478 (EGFR), 100nM PP2 (Src); 100nM Raf kinase inhibitor 1(R-1, c-Raf), 1μM H89 (PKA), 100nM PKA inhibitor 14-22 amide (PKi), 250μM SQ22536, (SQ,
Adenylate cyclase; 1μM Bisindolylmaleimide I (Bis, PKC), 100nM SB203580 (p38). To inhibit GPCR signaling, cell were pretreated for 24h with100 ng/ml pertussis toxin (Ptx, Enzo Life Sciences, Plymouth Meeting, PA).

Assessment of cell proliferation by BrdU immunostaining - Cells plated in 8-well or 4-well chamber slides (5x10⁴ or 1x10⁵ cells per well) were treated for 24h with INGAP, EGF or Ex 4, as described above, and 50μM BrdU was added during the last 3 hours of treatment. Cells were washed with PBS and fixed in Methanol for 10 min at -20C. Immunostaining for BrdU was carried out using mouse anti-BrdU antibody (Roche) following the manufacturer’s protocol. This was followed by detection with secondary, HRP conjugated antibody (broad spectrum, Histostain-Plus) and AEC chromogen (both from Zymed Laboratories). Slides were counterstained with hematoxylin. BrdU-positive and negative nuclei were counted (total 200 per well) and the percentage of BrdU-positive nuclei was calculated.

Western blot analysis- Following treatments, cells were placed on ice, washed with PBS and solubilized in lysis buffer (Cell Signaling, Inc., Beverly, MA), containing 2.5mM Na₄P₂O₇, 1 mM Na₃VO₄ and Complete protease inhibitor cocktail tablet (Roche). Equal amounts of protein (20-50μg, measured with DC Protein assay (Bio-Rad)) were resolved by 10 % SDS-PAGE, followed by transfer onto Nitrocellulose membrane (Bio-Rad) at 250mA for 90 min and analyzed with different antibodies. Anti- Erk1/2 (MAPK 44/42) and anti-phospho Erk1/2 (Thr202/Tyr204), anti- p38 MAPK and anti- phospho-p38 MAPK (Thr180/Tyr182), anti-C-Raf and anti- phospho-C-Raf (Ser338), anti- phospho-(pan)-PKC (γThr514), rabbit polyclonal antibodies were purchased from Cell Signaling. Following primary antibody incubation, blots were washed and then incubated in a secondary, anti-mouse or anti-rabbit HRP-conjugated antibody (Cell Signaling), washed and developed using the ECL system (GE Healthcare). To
analyze expression of several proteins on the same blot, membranes were first incubated with phospho-antibodies followed by stripping (0.2M Glycine, 0.1%SDS, 0.05% Tween20, pH2.2) prior to probing with corresponding non-phospho primary antibodies.

Ras activation and phospho-Akt(Ser473) ELISA - Ras-GTP and Akt activation in INGAP-stimulated RIN-m5F cells were analyzed using Ras activation and phospho-Akt(Ser473) ELISA kits purchased from Millipore. 1 x 10^6 cells were plated in 60mm plates for 48 hours followed by a 24-h starvation in serum-free medium. Cells were treated with the growth factors at 37°C, for the times indicated. Plates were then placed on ice and washed with ice-cold PBS prior to cell lysis in 150 μl of Mg+ lysis buffer containing a cocktail of protease inhibitors (NEB). 10 μl of cell lysates were used for Ras-GTP ELISA and the readings were normalized by the amounts of protein (DC protein assay, Biorad).

PKC and PKA kinase activity assay - Cell lysates prepared as for Western blots and containing 5-10 μg of crude protein per sample were assayed by ELISA using PKA- and PKC Kinase Activity Assay kits purchased from Assay Design (Ann Arbor, MI), according to the supplied protocol and normalized to total protein.

Visualization of fluorescent rINGAP and INGAP-P - 100μg of rINGAP were labeled with DyLight-488 or DyLight-594 (ThermoScientific) as specified in the instructions. INGAP-P was labeled with either 5-FAM or FITC during the synthesis at the Sheldon Biotechnology Centre (McGill University, Montreal) or Canpeptide (Ponte Clair, Quebec). Fluorescent rINGAP (50 nM) or INGAP-P (8.35-16.7 μM) were added to RIN-m5F cells grown in glass chamber slides (Beckton-Dickinson or Lab-Tek), for various intervals followed by washing with PBS and fixation in 4% paraformaldehyde. Slides were mounted using VectaShield medium (Vector) or Prolong Gold (Invitrogen) with DAPI for counterstaining of nuclei and examined under confocal
microscope Zeiss LSM 510 or Olympus FV10i. For live confocal imaging cells were grown in
Nunc™ chambered coverglass slides (ThermoScientific). Nuclei were stained with 0.01% DAPI
prior to incubation with INGAP followed by washing. Live imaging was carried out at 37°C and
5% CO2.

To study INGAP internalization, Cholera Toxin, subunit B (CTB, AlexaFluor-594, 5 μg/ml)
Transferrin (25μg/ml, Texas Red), and LysoTracker Red DND99 (LT, 50nM, all from
Invitrogen) were used in co-migration assays with DyLight-488 rLINGAP and FAM-INGAP-P.
To assess INGAP colocalization with early endosomes, fixed
0.1% Triton x100 for 10min, blocked in 5% goat serum and probed with anti-EEA1 rabbit
primary antibody (Abcam, 1:200) overnight, at 4 C followed by the secondary donkey anti-rabbit
DyLight594-conjugated antibody (1:500) for 1h at room temperature. Cells incubated with
DyLight-594-labeled rLINGAP were probed with anti-Clathrin and anti-Caveolin rabbit
antibodies (Abcam) followed by FITC-labeled goat- anti-rabbit secondary antibody (Abcam).
Nuclei were counterstained with DAPI included in the mounting medium (Prolong Gold,
Invitrogen). The following inhibitors of endocytosis were used: Dansylcadaverine (100-300
μM), Filipin (1μg/ml), Cytochalasin D (25 μg/ml), all from Sigma, and Wortmannin (100nM,
Calbiochem).

Statistical analysis - Experiments were repeated at least three times. Results are expressed
as means ± SEM. Since most of our data were normalized to control values prior to calculation
of Fold Change, we could not assume that the data follow Gaussian distributions. Therefore, for
statistical analysis we employed the non-parametric Kruskal-Wallis test followed by Wilcoxon
sum-rank test for pair-wise comparison, using the SPSS software (IBM). A p-value of <0.05 was
considered significant.
Results

INGAP-P and rINGAP dose-dependently increase proliferation of RIN-m5F cells but with different molar efficiencies.

Although pancreatic ductal cells have been understood to be a particular target of INGAP (38, 42), a number of studies including the results of clinical trials suggest that β-cells are also responsive to INGAP stimulation in a number of ways including potentiation of glucose-stimulated insulin secretion and upregulation of the corresponding genes, as well as increase in cell viability and proliferation (1, 4, 7, 18, 34, 49, 57). There was no significant effect on insulin expression in our experiments on RIN-m5F cells, but we observed that both INGAP-P and rINGAP dose dependently induced BrdU incorporation after 24 h (Fig. 1A). The effect of rINGAP appeared to plateau at 1nM (1.64x increase) and so this dose was used throughout the study, whereas INGAP-P was used at 835nM (1.72x increase in BrdU). Similar increases in proliferation were observed for EGF (10ng/ml) and Ex4 (10nM), used as positive controls (17) (Fig. 1A).

The increase in BrdU incorporation was consistent with a rapid temporal activation of Erk1/2, observed between 1 and 15 min after addition of either rINGAP or INGAP-P (Fig. 1B, quantification is shown in Fig. 7C). These results show that both protein and peptide act in a similar manner but with different molar efficiencies (at least 100 fold). We therefore investigated next whether INGAP protein and INGAP-P interact differently with the cell surface and/or activate different signalling pathways.
Interaction of rINGAP with the cell surface is characterized by clustering and slow internalization

Using DyLight 488- labeled rINGAP, we observed binding to the cell surface of RIN-m5F cells within minutes of exposure (Fig. 2). Bound rINGAP forms small clusters and patches on the cell surface resembling the crosslinking of membrane multiprotein complexes described for other ligands (15, 31, 37, 45, 55). This is different from a homogenous staining exhibited by Cholera Toxin B (CTB, AlexaFluor 594) and Transferrin (Texas Red, both from Invitrogen) that were used as positive markers for caveolin and clathrin mediated endocytosis. rINGAP binding is observed both at 37°C and on ice (Fig. 2A-D), which is suggestive of a high affinity receptor.

Although first signs of internalization are observed after 15 min, the protein appears to remain clustered on the cell surface for several hours (Fig. 2 C-E), unlike Transferrin and CTB, which internalize within 1h (Fig. 2, C-D). After a 5h-incubation, most of the fluorescent label is seen inside of cells (Fig. 2E) and is partially co-localized with the lysosomal marker LysoTracker red. After 24h, all labeled rINGAP appears to internalize and associate with lysosomes, albeit partially, but showing no further binding to the cell surface (Fig. 2F).

Interestingly, in the chase experiments, when cells were exposed to DyLight488 rINGAP only for 1h, followed by washing and culture without rINGAP for 5 or 24 h, the amount of internalized rINGAP was not significantly lower than after continuous incubation (Fig. 2 G, H). This, together with the aforementioned observation, suggests that the available INGAP receptor pool is all ligand-bound within 1h and that the receptor turnover time probably exceeds 24h.

The lack of co-migration between rINGAP and CTB or Transferrin suggests that rINGAP is not internalized via either a clathrin- or caveolin- mediated pathway. This is in line with the results of immunostaining for clathrin and caveolin, showing no co-localization with rINGAP
Because of a long internalization time of rINGAP, usage of specific inhibitors of clathrin-mediated (Chlorpromazin, Dansylcadaverin) or caveolin-mediated endocytosis (Filipin, and β- methylcyclodextrin), as well as Dynasore (dynamin inhibitor), was not practical due to their fast developing cytotoxicity. We believe that the co-migration experiments, as well as the lack of co-localization are sufficient to rule out clathrin and caveolin-mediated endocytosis as a main internalization route for rINGAP. Besides, we found that rINGAP internalization is inhibited by Wortmannin (inhibitor of fluid-phase pinocytosis and PI3K) and by Cytochalasin D, (inhibitor of actin polymerization) (Fig. 3 C, D), which is suggestive of macropinocytosis as a major mechanism for rINGAP endocytosis (24). Further co-migration experiments with dextran and treatments with other inhibitors, e.g. amiloride (48), could be used to verify this possibility. Of note, no co-localization with the early endosomal marker EEA1 has been observed for rINGAP (Fig. 4, I), which may further support the macropinocytosis route of internalization.

INGAP-P is rapidly internalized into the cytoplasm without clustering on the cell surface.

In contrast to rINGAP, we did not observe any binding of FAM-labeled INGAP-P to the membrane. However, the labeled peptide was visible in the cytoplasm of RIN-m5F cells after 5 min of incubation reaching a plateau after 30 min (Fig. 4). As seen in Fig. 4C, INGAP-P appears to co-localize with early endosomes after 30min and then gradually migrates into the lysosomal compartment, co-localizing with LysoTracker red (Fig. 4, D, F).

Besides differences in the dynamics of cell binding and internalization, some other differences between the protein and peptide have been observed. For example, internalized INGAP-P appears to degrade faster, as shown in 24-h experiments with continuous and “chase”
incubations (Fig. 4 G, H). Also, internalization of INGAP-P was inhibited on ice or by pre-incubation with the caveolae inhibitor Filipin (Fig. 5A, B), suggesting that this process might be mediated by caveolae/lipid raft endocytosis. Inhibitor of clathrin-dependent endocytosis Dansylcadaverine did not have a significant effect (Fig. 5C). On the other hand, INGAP-P internalization is inhibited by a 15min pre-incubation with cytochalasinD, resulting in formation of small clusters on the cell surface (Fig. 5D). This suggests that actin filaments are involved in the process of INGAP-P internalization. However, it’s unlikely to be macropinocytosis, as Wortmannin did not appear to have inhibitory effect on this process (Fig. 5E).

To investigate whether rINGAP and INGAP-P act via the same receptor, both DyLight488-rINGAP and FAM-INGAP-P were used in competition experiments with 20x molar excesses of the unlabeled protein and peptide. The results show that internalization of the protein is partially inhibited by cold protein and the peptide by cold peptide but they don’t appear to inhibit each other (Fig. 6), at least at the given concentration, which suggests that they likely do not bind the same receptor.

**Signaling pathways leading to Erk1/2 phosphorylation by both rINGAP and INGAP-P involve Ras-Raf activation.**

Activation of Erk1/2 may be mediated by a number of signaling cascades initiated at the cell membrane level by receptor tyrosine kinases (RTK) or by different classes of G-protein coupled receptors (GPCR). These signaling cascades include the PKC, PKA, PI3K or Ras/Raf-dependent pathways (30, 35, 46). Since the nature of the INGAP receptor is unknown, we screened for both RTK and GPCR- initiated signaling events using phospho-specific antibodies and pharmacological inhibitors of all the above-mentioned pathways. For comparison we used EGF
(10ng/ml) and Ex-4 (10nM), which was found to be mitogenic for RIN-m5F cells at the indicated concentrations (Fig. 1A). Because EGF signals through the classical RTK pathway and Ex-4 is an agonist of the G-protein coupled GLP-1 receptor (17), such a comparison may provide important clues to how INGAP works.

Activation of low molecular weight Ras family GTPases is the first key event in the signaling through RTKs, such as EGFR. It became apparent, however, that the mechanisms of MAP kinase activation by GPCRs may also include Ras activation by cross-talk between GPCRs and RTKs, e.g. transactivation of EGFR shown for several GPCR ligands, including GLP-1 (8, 30). In keeping with this notion, our results show a rapid Ras activation by both INGAP-P and rINGAP (Fig. 7A) that precedes phosphorylation of c-Raf (Fig. 7B) and Erk1/2, which peaks at 10min (Fig. 7C).

Since INGAP-P has been previously shown to activate the PI3K/Akt signaling pathway (5, 25), and because this pathway can be involved in cell proliferation, we measured phospho-Akt (Ser473) in a time course experiment and observed a weak increase (not statistically significant) by INGAP-P at 30 min, but not by rINGAP (Fig. 7D). In contrast, both EGF and Ex-4 induced a transient Akt activation at 1min, which preceded such of Erk1/2 (Fig. 7E). This is in line with previous studies showing that GLP1 and EGF-like ligands stimulate proliferation in beta cells via activation of PI3K/Akt pathway (8, 9). Accordingly, more late activation of Akt (at 30min) than Erk1/2 (10min) by INGAP-P suggests that the PI3K signaling is not involved in Erk1/2 phosphorylation in RIN-m5F cells. The fact that Akt does not seem to be activated by rINGAP indicates that signaling events upstream of Ras–Raf–Erk activation may vary between INGAP-P and rINGAP. Of note, we did not observe significant activation of either p38 MAPK (Western
blot), or PKA (ELISA), or PKC (Western blot and ELISA) by either protein or peptide (data not shown).

Pharmacological inhibition of signaling pathways implicates GPCR in mitogenic effects of INGAP on RIN-m5F cells.

To investigate signaling events implicated in INGAP induced proliferation, we employed specific pharmacological inhibitors of Raf (Raf inhibitor 1), PI3K (Wortmannin), PKC (Bis), PKA (H89, PKi), Adenylate cyclase (SQ22536), Src (PP2) and EGFR (AG1478). In addition, Pertussis toxin (Ptx) was used to examine whether INGAP actions were mediated by a GPCR. The effectiveness of these inhibitors was judged by Erk1/2 phosphorylation after 10 min of treatment with INGAP or EGF or Ex-4(Fig. 8).

As shown in Fig. 8A, INGAP-P- and rINGAP-induced activation of Erk1/2 was inhibited by ~40% after a 24h exposure to Ptx, but not affected by AG1478 (Fig. 8B). This suggests that INGAP likely signals through a GPCR but that this signaling does not involve the EGF receptor, as has been previously shown for GLP-1 (8). Ptx also inhibited early Ras activation induced by INGAP or EGF or Ex4, (Fig. 9) which further supports the idea that INGAP signals via a GPCR-Ras pathway. Consistent with the previous implication of Ras-Raf signaling, pretreatment with Raf kinase inhibitor 1 reduced Erk1/2 activation (Fig. 8B) by all growth factors tested. Interestingly, Src inhibitor PP2 was effective for rINGAP, but not for INGAP-P (Fig. 8B).

Further highlighting the differences in signaling between the protein and peptide, inhibition of PKC had a stimulatory effect on rINGAP-induced Erk1/2 activation, while no such effect was seen for INGAP-P (Fig. 8B).
For comparison, Erk1/2 activation by EGF was inhibited with AG1478, Wortmannin, PP2, H89, Ptx, and Raf inhibitor 1, whereas only Raf inhibitor 1 and Ptx had an effect on Ex-4. As expected, PD98059 was an effective inhibitor for all growth factors tested.

Discussion

Based on the original studies of discovery and cloning of INGAP (39), it has been believed that peptide\textsuperscript{104-118} (INGAP-P) is an active center of the protein, as it had essentially the same effects on target tissues. Since then, research has focused on the peptide, as a more clinically relevant compound (28-32), which is now in Phase II clinical trials (18). However, the peptide has a limited stability and has to be administered in high doses. In an effort to improve INGAP-P efficacy, we searched for clues in the full-length INGAP protein, which we recently cloned and which displayed a much greater stability and at least 100 times higher molar efficiency in inducing \textit{in vitro} regeneration of functional human islets from dedifferentiated, islet-derived duct-like structures (2). Likewise, significant differences in effective concentrations of INGAP peptide and protein have been observed for both proliferative (ARIP cells, hamster ductal explants (39)) and differentiating effects (human ductal cells HPDE, Assouline-Thomas, unpublished observations)). In the current work, we also demonstrate that rINGAP is more efficient on a molar basis (over 100 x) than INGAP-P in stimulation of proliferation in RIN-m5F cells, although both ligands activate in principle the same Ras-Raf-Erk pathway.

The data suggest that the difference in molar efficiency is most likely based on how rINGAP and INGAP-P interact with the cell surface. rINGAP binds RIN-m5F cells in a manner consistent with clustering of the ligand-receptor complexes, which may also include other membrane proteins, such as integrins, proteoglycans and various glycoconjugates, and which
may contribute to a higher intensity of the initiated signaling (47). Formation of such complexes could also explain delayed internalization of rINGAP that probably occurs by macropinocytosis, as indicated by the inhibition experiments with Wortmannin and Cytochalasin D. At present, it is unclear whether rINGAP internalization plays a role in the downstream signaling or whether it only serves the purpose of ligand-receptor degradation once the signalling cascade is initiated. Similar internalization kinetics have been described for hepatoma-derived growth factor (HDGF), which binds its receptor and heparan-sulphate proteoglycans (55). As shown for HDGF, receptor binding initiates downstream signalling, whereas the heparan-sulphate based internalization, also by macropinocytosis, modulates the strength of the related signalling processes. Such a possibility for INGAP should be explored once INGAP binding partners are identified.

In contrast, INGAP-P seems to interact with the membrane in a short-lived transient fashion, translocating into the cytoplasm in a few minutes. The observed sensitivity of this process to Filipin, an inhibitor of caveolae-based endocytosis and to Cytochalasin D, an inhibitor of actin assembly, but not to the inhibitors of clathrin-based endocytosis, or to Wortmannin implicates caveolae as the major route of internalization for INGAP-P.

In view of the observed differences, the main question is whether the peptide binds a specific receptor and whether this receptor is the same as for the protein. Although the lack of binding on ice may suggest against the receptor for INGAP-P, a number of studies show that low temperature may affect the peptide ligands conformation, which may in turn affect the binding (27). Another argument for a receptor is that the activation of Ras-Raf-Erk1/2 cascade by both INGAP-P and rINGAP is blocked by pertussis toxin, suggesting the involvement of a GPCR. As shown for PTH and PTH-related peptide, the same receptor may activate different signaling
pathways when activated by related but not identical ligands (16, 21). It is therefore possible that INGAP-P, as an active center of rINGAP, binds the same receptor, but due to different duration and conformational changes produced by the receptor-ligand interactions, the downstream signaling responses vary. However, it is also possible that INGAP-P and rINGAP bind and activate different receptors, especially because no competition between the two has been found in our experiments.

The lack of knowledge about INGAP receptor(s) complicates delineation of signaling mechanisms upstream of the Ras-Raf-Erk cascade. The results of screening with pharmacological inhibitors for most common transducing molecules, previously reported for INGAP-P signaling, such as PI3K, PKA, Adenylate cyclase (25, 51), and PKC show that these kinases are not involved in Erk 1/2 activation in RIN-m5F cells induced by either rINGAP or by INGAP-P. To the contrary, we observed a stimulatory effect of Bis (PKC inhibitor) on rINGAP induced P- Erk1/2. Together with sensitivity to Ptx, these data suggest that both ligands likely act via a G_{i}-protein coupled receptor(s) that does not activate PKC or adenylate cyclase (30). The proposed signaling pathway for rINGAP thus looks like GPCR – Src- (?)- Ras/Raf/Erk, whereas the pathway for INGAP-P remains less defined and may be described as GPCR- (?)-Ras/Raf/Erk.

This is the first study that compared INGAP protein and INGAP-P, using RINm5F cells as a model and focusing on the proliferative response and Erk1/2 activation. However, effects of INGAP-P on this cell line have been also studied by others reporting an increase in proliferation, as well as an upregulation of muscarinic M_{3} receptor after 72h, which was mediated by activation of NFkB (34). Of note, upregulation of M_{3} receptor by INGAP-P via NFkB, and the associated with that increase in insulin secretion, was also observed in MIN6 cells (34) and in
neonatal rat islets (5), thus implicating NFkB activation by INGAP-P as a common mechanism in insulin-secreting cells. Signaling events leading to NFkB activation are yet to be investigated. It would be interesting to determine whether rINGAP also activates NFkB and upregulates M₃ receptor in β-cell lines and pancreatic islets and whether it does so with a higher molar efficiency than the peptide.

A number of signaling pathways activated by INGAP-P have been reported, with PI3K-Akt pathway being the most common (5, 25). A rapid activation of Erk1/2 (MAPK3/1) by INGAP-P has also been shown in neonatal rat islets (5) and involvement of cAMP-PKA in the INGAP-P induced neurite outgrowth has been reported (51). Although our data do not indicate involvement of PI3K/Akt in the proliferative action of INGAP (Fig. 8), we did, however, observe weak Akt phosphorylation in cells treated with INGAP-P for 30 min, which comes later than the Ras-Raf-Erk activation. It can therefore be speculated that INGAP-P-induced PI3K/Akt signaling induces other responses in RINm5F cells, e.g. NFkB pathway, which should be investigated in future studies. Likewise, a more broad assessment of signaling activated by rINGAP has to be undertaken after a short-term and a long-term exposure. Inclusion of other cell types such as ductal cells or pancreatic islets into these studies is also needed to fully understand the potential and the mechanism of action of rINGAP and INGAP-P.

Taken together, our data show that both rINGAP and INGAP-P stimulate cell proliferation via Ras-Raf-Erk pathway and suggest involvement of a Gi-protein coupled receptor(s). The major differences between the two are related to cell binding and internalization, which we believe are responsible for a much lower molar efficiency of the peptide. These data suggest that modifications of the peptide leading to a more rINGAP-like cell binding might be an interesting approach to improve INGAP-P efficacy as a therapy for diabetes.
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16. Dean T, Vilardaga JP, Potts JT, Jr., and Gardella TJ. Altered selectivity of parathyroid hormone (PTH) and PTH-related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. Mol Endocrinol 22: 156-166, 2008.


Figure 1. Effect of INGAP on proliferation in RIN-m5F cells. A. INGAP increases BrdU incorporation. RIN-m5F cells were treated with indicated amounts of INGAP-P or rINGAP for 24h in chamber slides. Exendin 4 (Ex4) and EGF were used as positive controls. Each dose was compared to the control and the data were analysed as a ratio of BrdU(+) cells (%) in INGAP-treated to untreated control. Shown are means ± S.E. of at least three independent experiments (* p<0.05, † p<0.01, compared to untreated control; Kruskal-Wallis and Wilcoxon sum-rank test). B. INGAP induces phosphorylation of Erk1/2 in RIN-m5F. RIN-m5F cells (1x10⁶) plated in 60mm tissue culture plates were treated with INGAP for the times indicated. Blots (30μg protein) were probed with anti–Erk1/2- Phospho (Thr202/Tyr204) antibody, followed by stripping/reprobing with anti-total Erk1/2 antibody (Cell Signaling) and quantified by densitometry, using ImageJ software. The results of quantification are presented in Fig 7C.

Figure 2. Binding of rINGAP is characterized by capping on the cell surface at 37ºC or on ice and by slow internalization. A. Cells were pre-chilled on ice for 15 min and incubated with DyLight488-rINGAP and CTB (AlexaFluor-594, 5 μg/ml, Invitrogen) for 30 min. B. same with Transferrin (25μg/ml, Texas Red, Invitrogen). C, D. 1h incubation with CTB and Transferrin, respectively, at 37ºC. E, F. Cells were incubated for 5h or 24h with labeled rINGAP and co-stained with 50nM LysoTracker Red DND99 (LT, Invitrogen) for the last hour. G, H. Chase experiment: Cells were incubated with rINGAP for 1h followed by washing and a chase period of 5h (G) or 24h (H) without presence of labeled INGAP. LysoTracker Red DND99 (50nM) was added 1 h prior to fixation in 4% PFA. I. Negative control. Bars are 20μ. J-K. Cells
were incubated with DyLight-594-labeled rINGAP for 1 min (J) or 15 min (K) fixed and probed with anti-Clathrin and anti-Caveolin rabbit antibodies followed by FITC-labeled goat-anti-rabbit secondary antibody. L. No primary antibody control to J and K. Nuclei were counterstained with DAPI included in the mounting medium (Prolong Gold, Invitrogen). Images were taken with Olympus FV10i (A-I) and Zeiss LSM-510 (J-L) confocal microscopes.

**Figure 3.** Binding and internalization of fluorescently labeled rINGAP is partially inhibited by 100 nM Wortmannin and Cytochalasin D, suggestive of macropinocytosis. RIN-m5F cells plated in chamber slides were incubated for 5 h with 50 nM DyLight 488-rINGAP: A. without inhibitors; B. in the presence of Cytochalasin D (25 μg/ml), or C. Wortmannin (100 nM); D. negative control. Nuclei were counterstained with DAPI included in the mounting medium (Prolong Gold, Invitrogen). Images were taken with Olympus FV10i confocal microscope.

**Figure 4.** FAM-labeled INGAP-P is rapidly internalized into the cytoplasm of RIN-m5F cells. Cells grown in chamber slides were treated with FAM-labeled INGAP-P for the times indicated and fixed with 4% PFA. A, B, D and F. Cells were stained with Lysotracker for 1 h, as for Fig. 2. C, E, Fixed cells were costained for EEA1 and visualized with secondary donkey anti-rabbit DyLight594-conjugated antibody. Nuclei were counterstained with DAPI included in the mounting medium (Prolong Gold, Invitrogen). Images were taken with Olympus FV10i confocal microscope. G. A continuous incubation with FAM-INGAP for 24 h. H. Chase experiment: Cells were incubated with FAM-INGAP for 1 h followed by washing and a chase period of 24 h, without presence of labeled INGAP. LysoTracker Red DND99 (50 nM) was added.
1 h prior to fixation in 4% PFA. I. No colocalization with EEA1 was observed for rINGAP (2h). Nuclei were counterstained with DAPI included in the mounting medium (Prolong Gold, Invitrogen). Images were taken with Olympus FV10i confocal microscope.

Figure 5. Internalization of FAM-INGAP-P is inhibited on ice, by Filipin and Cytochalasin D but not by Wortmannin or Dansylcadaverine. Cells grown in chamber slides were treated with FAM-labeled INGAP-P (16.7 μM) for 1h under various conditions: A. on ice; B. in the presence of 1μg/ml Filipin; C. Dansylcadaverine (300 μM). D. Cytochalasin D (25 μg/ml); E. Wortmannin (100nM). F DMSO 1μl (same volume as for the inhibitors). Cells were fixed and counterstained with DAPI as described above and imaged using either Zeiss LSM 510 (A,B) or Olympus FV10i(C-F).

Figure 6. Molar excess competition assay for binding and internalization of fluorescently labeled rINGAP and INGAP-P. RIN-m5F cells plated in chamber slides were incubated with FAM-INGAP-P for 1h (left panel) or with DyLight-488 rINGAP for 5h (right panel) A, B. no inhibition; C,D. with 167 μM INGAP-P (10x molar excess); E,F. with 1μM rINGAP (20x molar excess).

Figure 7. Involvement of Ras – Raf –Erk1/2 activation in signaling events induced by INGAP-P and rINGAP. Cell lysates collected in the time course experiments with rINGAP or INGAP-P were used for multiple analyses by ELISA (Ras-GTP and Akt(Ser473) (Millipore) and by Western blot (Phospho-Erk1/2, Phospho-c-Raf). The data are presented as a Fold Change over the 0min time point, which equals 1 and is shown as a dotted line in all charts. A. Ras
activation was measured by Ras-GTP ELISA as described in Materials and Methods (* p<0.05, † p<0.01). B. c-Raf phosphorylation, measured by Western blot /densitometry (ImageJ) as a ratio of Phospho to total c-Raf. The changes were not found to be statistically significant. C. Quantification of relative Erk1/2 phosphorylation measured by Western blot /densitometry (ImageJ) as a ratio of Phospho-Erk1/2 to total Erk1/2. (* p < 0.05, compared to time 0). D. Changes in Akt phosphorylation in RIN-m5F cells treated with INGAP-P, rINGAP. E. Phosphorylation of Erk1/2 and Akt induced by EGF and Ex-4 quantified as in C and D (* - p < 0.05 for P-Erk1/2; §- p< 0.05 for P-Akt).

Figure 8. Effect of pharmacological inhibitors on Erk1/2 phosphorylation by INGAP, EGF and Ex-4. RINm5F cells grown in 60mm plates were pretreated for 30-40 min with the indicated inhibitors, except for Ptx (24h pretreatment). After a 10min treatment with growth factors, cells were placed on ice and lysed. 30 μg of proteins were probed for Phospho-Erk1/2. Data are shown as a ratio of Phospho-Erk1/2 to total Erk1/2 relative to the “no inhibitors” group (=1, shown as a dotted line). A. Inhibitors of GPCR (Ptx, 100ng/ml), adenylate cyclase (SQ, 250μM) and PKA (PKi, 100nM; H89, 1μM) or DMSO as a vehicle control. B. inhibitors of PKC (Bis, 1μM), PI3K (Wm, 100nM), Src (PP2, 100nM), EGFR (AG, 100nM), MEK (PD) and c-Raf (Raf-1, 100nM), (* - p < 0.05, § - p<0.01, compared to “no inhibitor” group for each growth factor treatment).

Figure 9. Inhibition of GPCR signaling results in diminished Ras activation. RIN-m5F cells grown in 60 mm plates were pretreated with Ptx for 24h prior to addition of growth factors for 1,3,5 and 10 min. Control cells were treated with growth factors (no Ptx) for the same
intervals. Cells were harvested in Mg+ lysis buffer and subjected to the Ras-GTP ELISA, as
described in Materials and Methods. The results are shown as a ratio of Ptx- treated to control
per each time point and are means ± S.E. of at least three independent experiments (* - p < 0.05,
compared to control, which are equal 1 and are shown as a dotted line ).


Figure 1

A

Fold Change in BrDu labeling vs control

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B

INGAP-P (835nM)    rINGAP (1nM)

P-Erk1/2

Erk1/2
Figure 3
Figure 4
Figure 6
Figure 8

A

B
Figure 9

![Graph showing relative Ras activity in Ptx treated cells with EGF, Ex-4, INGAP-P, and rINGAP.](image)