Insulin signal transduction in rat small intestine: role of MAP kinases in expression of mucosal hydrolases

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Marandi, Soheila, Nadine De Keyser, Alain Saliez, Anne-Sophie Maernoudt, Etienne Marc Sokal, Catherine Stilmant, Mark H. Rider, and Jean-Paul Buts. Insulin signal transduction in rat small intestine: role of MAP kinases in expression of mucosal hydrolases. Am J Physiol Gastrointest Liver Physiol 280: G229–G240, 2001.—The postreceptor events regulating the signal of insulin downstream in rat intestinal cells have not yet been analyzed. Our objectives were to identify the nature of receptor substrates and phosphorylated proteins involved in the signaling of insulin and to investigate the mechanism(s) by which insulin enhances intestinal hydrolases. In response to insulin, the following proteins were rapidly phosphorylated on tyrosine residues: 1) insulin receptor substrates-1 (IRS-1), -2, and -4; 2) phospholipase C-isoenzyme-γ; 3) the Ras-GTPase-activating protein (GAP) associated with Rho GAP and p62Src; 4) the insulin receptor β-subunit; 5) the p85 subunits of phosphatidylinositol 3-kinase (PI 3-kinase); 6) the Src homology 2 α-collagen protein; 7) protein kinase B; 8) mitogen-activated protein (MAP) kinase-1 and -2; and 9) growth receptor-bound protein-2. Compared with controls, insulin enhanced the intestinal activity of MAP kinase-2 and protein kinase B by two- and fivefold, respectively, but did not enhance p70/S6 ribosomal kinase. Administration of an antireceptor antibody or MAP-kinase inhibitor PD-98059 but not a PI 3-kinase inhibitor (wortmannin) to sucklings inhibited the effects of insulin on mucosal mass and enzyme expression. We conclude that normal rat enterocytes express all of the receptor substrates and mediators involved in different insulin signaling pathways and that receptor binding initiates a signal enhancing brush-border membrane hydrolase, which appears to be regulated by the cascade of MAP kinases but not by PI 3-kinase.

insulin receptor substrates; phosphotyrosine proteins; signal transduction

Although the intestinal mucosa is not a classic target tissue for insulin, accumulating evidence (2, 3, 6–11) indicates that insulin determines important physiological effects on intestinal growth, cell maturation, and enzyme expression in several mammalian species. The onset of weaning (day 14–17) in the suckling rat is a critical period during which immature enterocytes exhibit elevated responsiveness to the hormone. At this time, plasma insulin levels rise markedly (3), whereas milk-borne insulin is still active (10), allowing optimal interaction of the hormone with intestinal insulin receptors (IR), which are located on both endoluminal and basolateral membranes of the cell (8). After weaning, the twofold decrease in IR concentration is associated with a reduction in responsiveness of mature enterocytes to the hormone (7, 8). Our recent studies (9, 11) suggest that the premature induction of sucrase-isomaltase (SI) is triggered by the binding of the hormone to the extracellular α-receptor subunit, allowing autophosphorylation of the tyrosine kinase intrinsic to the juxtamembrane and cytoplasmic domains of the β-receptor subunit (9). Downstream transduction of the signal into the cell is associated with an increase in endoluminal polyamine uptake and leads to the final activation of the SI gene promoter with a dose-dependent accumulation of SI mRNA, independent of the mitogenic effects of the hormone (11).

In response to IR activation, several receptor substrates (IR substrate-1 (IRS-1), IRS-2, IRS-4, and Src homology 2 α-collagen (She)) and tyrosine-phosphorylated proteins associated through Src homology 2 domains (SH2 domains) have been purified and sequenced from insulin target cells cultured in vitro (14, 18, 34, 35, 43, 46, 47, 52–54, 56, 57). However, the insulin signal transduction in the rat small intestine has not been investigated so far. In addition, the specific pathway by which insulin stimulates brush-border membrane (BBM) hydrolases remains unknown. Using the intact live animal under conditions relevant to normal insulin responses, the objectives of our study were 1) to analyze the nature of IRS and phosphorylated proteins involved in insulin signal transmission at the level of the small intestine and 2) to approach the signal pathway activating BBM hydrolases.

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MATERIALS AND METHODS

Reagents. Phenylmethanesulfonic acid, dithiothreitol, HEPES, sodium salt, SDS (highest purity grade), 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, Triton X-100, leupeptin, pepstatin, aprotinin, EDTA, Wortmannin, PD-98059, and myelin basic protein were purchased from Sigma Chemical (Bornem, Belgium). Human recombinant insulin (Actrapid HM) was from Nordisk ( Bagsvaerd, Denmark), and 125I-labeled protein A and molecular weight standards (CFA 626) were from Amersham Laboratories (Little Chalfont, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad (Nazareth, Belgium), and protein A-Sepharose 4B was from Pharmacia LKB Biotechnology. Polyclonal antibodies (PY20) recognizing tyrosine-phosphorylated proteins were purchased from Transduction Laboratories (Cambridge, UK). Specific polyclonal antibodies recognizing IRS-1, IRS-2, phospholipase C-isoenzyme-γ (PLC-γ), Shc, GTPase-activating protein (GAP), protein tyrosine phosphatase-2 (SYP), growth receptor-bound protein-2 (Grb2), the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), and mitogen-activated protein (MAP) kinases were from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA). Anti IRS-4 polyclonal antibody was produced by repeated rabbit immunizations with a peptide (31 amino acids) corresponding to the COOH-terminal sequence of human IRS-4. RPN 538, a monoclonal antibody recognizing epitopes of the α-extracellular subunit of the IR, was purchased from Amersham (Brussels, Belgium). 2C4, a monoclonal antibody that recognizes a 60-kDa receptor substrate associated with GAP in Chinese hamster ovary cells (CHO) (expressing IR (CHO-IR) (18) and immunoprecipitates a docking protein (p62αβγ) associated with Ras-GAP in v-abl-transformed murine precursor B cells (54), was a generous gift of Dr. Richard Roth (Stanford University, Stanford, CA). The anti-protein kinase B (PKB) polyclonal antibody recognizing PKB-α and PKB-λ isoforms was raised in rabbits against the COOH-terminal peptide (FPQFYSASSTA) of rat PKB.

Animals. All procedures were approved by the National and University Animal Care Committees (Fonds de la Recherche Scientifique Médicale and Université Catholique de Louvain, Brussels, Belgium). Litters of Wistar rats, acclimated to standard conditions of room temperature, light-dark cycles, and feeding schedules, were used. During the nursing period, pups remained with their mothers in polystyrene cages and had free access to milk. Suckling rats were killed at postpartum. Adult rats (180 g) were fed a pelleted diet (AO3 UAR, Villemoisson-sur-Orge, France) ad libitum. To identify receptor substrates, animals were injected under ether anesthesia after 12–24 h of fasting. The femoral vein was exposed after a small incision, and normal saline (0.9% NaCl) with (10−4 mol/l) or without insulin was slowly injected (0.1 ml/rat). After infusion, the animal was killed and the small intestine was excised rapidly, trimmed of fat and mesentery, and rinsed with ice-cold saline. After being opened longitudinally, the mucosa was scraped on ice between glass slides, weighed, and used for protein extraction or stored at −170°C in liquid nitrogen.

Treatment schedules. To approach the insulin signaling pathway that activates BBM hydrolases, antibodies recognizing extracellular epitopes of the IR (α-subunit) and key enzyme inhibitors (wortmannin and PD-98059) were injected into suckling pups for 48–72 h. To equalize conditions of nursing and feeding, dams were reduced to six pups per lactating mother. Human insulin (Actrapid MC, Novo Industries, Brussels, Belgium) was injected intraperitoneally to sucklings from day 11 to day 14 postpartum (7:30 AM and 7:30 PM) at a dose of 5 mU/g body wt−1·day−1 in a volume of 100 μl. Control groups were treated with equivalent volume of 0.9% saline. A monoclonal antibody recognizing epitopes of the extracellular α-IR subunit (RPN 538, Amersham, Gent, Belgium) was injected intraperitoneally following the same schedule as insulin at 20 μg protein/dose twice daily. To inhibit PI 3-kinase, wortmannin was injected intraperitoneally at a dose of 5 μg/g body wt−1·day−1 1 h before the administration of insulin (5 mU/g body wt) from day 11 to day 14 postpartum. Control animals received insulin alone.

To inhibit MAP kinase kinase and MAP kinases, PD-98059 was injected intraperitoneally into sucklings at a dose of 2 μg/g body wt twice daily from day 12 to day 14 postpartum. Insulin was injected 1 h after administration of the inhibitor at a dose of 5 mU/g body wt. Control rats received insulin according to the same schedule.

Extraction of phosphotyrosine proteins. We used the method of Rothenberg et al. (41) with slight adaptations. Briefly, samples of intestinal mucosa were homogenized for 1 min in a solubilization buffer (1:5 vol/vol) maintained at 100°C in a water bath (2 min) with an Ultraturrax generator (Janke and Kunkel, Staufen, Germany) operated at maximum speed. The solubilization buffer was composed of 2% SDS, 100 mmol/l HEPES (pH 7.8 at 22°C), 10 mmol/l EDTA, 100 mmol/l NaCl, and 50 mmol/l dithiothreitol. The homogenate was heated further to boiling with gentle stirring for 2 min and then left to cool to 22°C.

After centrifugation for 2 h at 18°C in a Beckman type 35 rotor ultracentrifuge (143,000 g), the supernatant was acidified with 100% TCA, added slowly dropwise at 22°C with vigorous stirring to a final concentration of 10%. The mixture was then cooled at 4°C overnight. Under these conditions, protein and nucleic acids form a copious, flocculent, pink precipitate, whereas SDS remains largely soluble. The precipitate was collected by centrifugation in a Sorvall RC-5B centrifuge at 4°C for 10 min. The precipitate was washed once with 25 volumes of 1% TCA at 4°C for 10 min, and the TCA was then extracted by six washes, each with 25 volumes of ethanol and diethyl ether (1:1 vol/vol) at 4°C. The precipitate was dried in vacuo (Speed Vac) for 12–18 h and pulverized thoroughly to a fine powder. In this form the extracted proteins could be stored for at least 1 year at −170°C without apparent degradation or significant loss of phosphotyrosine content.

Immunoprecipitation. For immunoprecipitation of proteins, 0.05 g of dry tissue powder (≥15 mg proteins) was dissolved in 1 ml 0.1 N NaOH with vigorous stirring at 22°C for 5 min. The resulting solution was then neutralized rapidly to pH 8 with 2 volumes of 100 mmol/l Tris-HCl, 1 mmol/l EDTA, and a cocktail of protease inhibitors including 1 mmol/l phenylmethanesulfonic acid, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. The slightly turbid solution was clarified by a 0.45-μm pore diameter cellulose/polyvinyl chloride filter (Millex-HA, Millipore). Protein concentration was measured using the Lowry assay (31). Poly- or monoclonal antibodies were added to a final concentration of 1–4 μg IgG/ml and incubated at 4°C for 4 h. Immune complexes were then absorbed to protein A-Sepharose 4B beads (25 μl of a 50% bead slurry/ml of extract) for 12 h at 4°C with gentle rotation. The immune complexes were washed twice by resuspension and brief centrifugation in 1 ml of wash buffer containing 1% Triton X-100, 0.1% SDS, 100 mmol/l NaCl, and 50 mmol/l Tris, pH 7.3, at 22°C and once more in the same buffer lacking NaCl. After aspirating the excess wash buffer, the immunoprecipitated proteins were solubilized in 30–50 μl of Laemmli’s buffer (23) and boiled at 100°C for 5 min before being layered onto gel slots.
Electrophoresis and immunoblotting. Immunoprecipitated proteins were separated by SDS-PAGE in 7.5% polyacrylamide gels as described previously (8, 9). Electrotransfer of proteins to PVDF membranes was performed for 90 min at 50 V as described by Towbin et al. (50). Nonspecific protein binding was reduced by preincubating the membrane overnight at 4°C in blocking buffer containing 5% BSA and 1% ovalbumin in TN buffer (10 mmol/l Tris, pH 7.2, and 0.9% NaCl). The membrane was then incubated with appropriate antibodies diluted in blocking buffer (0.5–2 μg/ml) for 2 h at 22°C and washed twice for 10 min in TNT buffer (TN buffer plus 0.1% Tween), once for 10 min in TN buffer containing 0.05% Nonidet P-40, and twice for 10 min each in TNT buffer. The blots were then incubated with 50 μCi of 125I-labeled protein A (Amersham) in 10 ml of blocking buffer for 1 h at 22°C and then washed again as described above. Bound antiphosphotyrosine or specific antibodies were detected by autoradiography using 24 × 30 cm Fuji film (St. Nicolas, Belgium) at −70°C for 72 h as described previously (8, 9). Band intensities were quantified by optical densitometry using an image densitometer (CS-690, Bio-Rad). Relative intensity was expressed in arbitrary optical density units (OD units), whereas relative abundance was expressed in arbitrary volume units (OD × mm²).

Enzyme assays. To measure the intestinal activity of PKB, MAP kinase-2, and p70/S6 kinase, mucosal homogenates (1.3 vol/vol) were prepared from insulin-treated rats and controls under nonadenaturing conditions. Enzymes were immunoprecipitated with the corresponding specific antibodies bound to protein-A-Sepharose 4B beads in a buffer containing a cocktail of antiproteases. Enzyme activities were determined by the phosphorylation of three different peptides used as substrates in the presence of [γ-32P]ATP-Mg (sp act >5,000 Ci/mmol, Amersham). For MAP kinase-2, the substrate was the myelin basic protein (phosphorylated on serine/threonine residues), for PKB, it was a synthetic peptide called MR-15 (RPRAATF), and for p70/S6 kinase, it was the peptide MR-4 (RRLESSLRA). Blanks were handled exactly like samples, except that the immunoprecipitated enzyme was omitted from the reaction. The activities were linear for up to 20 min of incubation time (30°C) and were proportional to the amount of enzyme used in the reaction. Protein kinase activity was expressed in picomoles of substrate phosphorylated per minute per gram of mucosa.

Sucrase, lactase, and maltase activities were assayed in BBM samples by standard methods (9, 11). Activities were expressed as micromoles of substrate hydrolyzed per minute per milligram of BBM protein.

Calculations and statistics. All data are given as means ± SD except enzyme activities, which are expressed as means ± SE. If not indicated, SD represents <10% of the mean. Differences between controls and insulin-treated animals were tested for statistical significance (P < 0.05) using the nonparametric Mann-Whitney U-test.

RESULTS

Identification of receptor substrates and insulin-elicited phosphotyrosine proteins in intestinal mucosal extracts. To identify IRS and phosphotyrosine proteins, we used the method of Rothenberg et al. (41) with minor modifications. Because phosphotyrosine proteins are susceptible to rapid phosphatase-mediated dephosphorylation both in vivo (24) and during extraction procedures (21), we rapidly homogenized intestinal mucosa from insulin-treated and control rats at 100°C in buffer containing 2% SDS and 50 mmol/l dithiothreitol. The final precipitate yielded 0.1 ± 0.06 g powder/g wet intestinal mucosa (n = 29) and 0.57 ± 0.09 g protein/g dry powder. We found no difference between the amount of protein extracted from the intestinal mucosa of insulin-treated rats and controls. In response to intravenous infusion of insulin (10⁻⁶ mol/l for 3 min), direct probing of mucosal extracts with antiphosphotyrosine antibodies (PY20) revealed that nine proteins (p165, p130, p120, p94, p85, p70, p60, p46, and p42) were rapidly phosphorylated on tyrosine sites (Fig. 1, left). In control animals, most of these proteins were undetected, whereas two (p130 and p60) showed only a weak signal. Densitometric measurements of each signal revealed that the following seven proteins had emerged by their signal intensity and relative abundance (in OD units × mm²): p165, p130, p120, p94, p85, p70, and p60 (Fig. 2A). After immunoprecipitation of protein extracts with antiphosphotyrosine antibodies (PY20) and Western blotting the membrane with the same antibody, p165 and p94 were detected again as single protein bands in insulin-stimulated rats without corresponding signal in control rats (Fig. 1, right). These proteins are most likely IRS-1 and IR β-subunit proteins. The same autoradiography shows a major complex of several proteins ranging from ~55 to 68 kDa, which were detected in both insulin-treated and control rats without difference in relative abundance, indicating the presence of phosphotyrosine proteins insensitive to insulin and likely the heavy chain of the antibody at 55 kDa. Because we (8) have recently identified in BBM of suckling rats a 60-kDa protein as a direct substrate of the IR, mucosal extracts from insulin-treated rats were immunoprecipitated with the same anti-p60 antibody (clone 2C4). This procedure failed to reveal this substrate in adult rat mucosal intestinal extracts (Fig. 1, right). To determine the nature of the p165 phosphotyrosine protein, protein extracts were immunoprecipitated with an anti-IRS-1 polyclonal antibody raised against the COOH-terminal peptide of rat liver IRS-1 (YASINFQKQ-PEDRQ) (46). After electrophoresis, PVDF membranes were probed with antiphosphotyrosine antibodies (PY20). Assays performed in adult rats after 3 min of insulin stimulation revealed a very weak signal (data not shown). Because the concentration of IR is higher in the small intestine of weanling rats than in adult rats (8) and the time of IRS-1 phosphorylation by the IR is very short (41), the experiment was repeated in weanling rats (day 23) stimulated for 1 min with insulin (10⁻⁶ mol/l). Figure 3, left, shows the detection in insulin-stimulated rats of two proteins migrating at ~185 and 165 kDa, respectively, whose signals were nearly absent in control rats, confirming that p165 is a phosphotyrosine protein corresponding to IRS-1 (41, 46). Because rat IRS-1 and mouse IRS-2 share up to 40% homology with 455 positive identities on 1,117 amino acid residues (46, 47) and the anti-rat IRS-1 polyclonal antibody used recognizes several motifs of the COOH-terminal domain of IRS-2 (according to the BLAST online search service of the National Center for...
Biotechnology Information), it is likely that the antibody coimmunoprecipitated both IRS-1 and IRS-2 and that the upper signal at 185 kDa corresponded to IRS-2, the reported molecular mass of IRS-2 (47). This was confirmed by immunoprecipitating protein extracts with an anti-IRS-2 antibody raised against a large fragment of the COOH-terminal domain of mouse IRS-2 (amino acids 976–1094) (47). As shown in Fig. 3,

Fig. 1. *Left*: autoradiography of phosphotyrosine proteins detected in intestinal mucosal protein extracts of adult rats after electrophoretic separation (7.5% SDS-PAGE) and Western blot with antiphosphotyrosine antibodies (PY20) (see MATERIALS AND METHODS). After 3 min of intravenous infusion of insulin (10^{-6} mol/l), 9 protein bands ranging from 42 to 165 kDa were detected in insulin-stimulated animals (INS+, lane 2), whereas in controls (INS−, lane 1) these proteins were either not or very weakly tyrosine phosphorylated. *Right*: mucosal protein extracts from insulin-stimulated animals (lane 4) or controls (lane 5) were immunoprecipitated with antiphosphotyrosine antibodies (PY20), and after electrotransfer, membranes were blotted with the same antibodies, as described in MATERIALS AND METHODS. Two major phosphotyrosine proteins of 165 [presumably insulin receptor substrate-1 (IRS-1)] and 94 kDa (presumably the β-subunit of the IR) were phosphorylated in response to insulin (lane 4) but were nearly undetected in controls (lane 5). Note a broad band of several phosphotyrosine proteins (ranging between 55 and 68 kDa) and most likely the heavy chain of the antibody seen in both insulin-stimulated (lane 4) and control animals (lane 5). Immunoprecipitation of mucosal extracts with anti-p60 (2C4) antibodies did not detect p60 phosphotyrosine protein in insulin-stimulated rats (lane 3).

Fig. 2. *A*: optodensitometric measurements of insulin-elicited phosphotyrosine proteins detected by Western blot using antiphosphotyrosine antibodies. (Fig. 1, *left*). Results are expressed in relative abundance (arbitrary optical density (OD) units × mm^2). I, insulin stimulated; C, controls. *B*: relative abundance of the α p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and of the p68-associated protein in mucosal protein extracts of insulin-treated rats and controls. The autoradiography showing the expression of these immunoprecipitated proteins is depicted in Fig. 5, *top left*. *C*: relative abundance of the 3 subunits (p66, p52, and p46) of Src homology 2 a-collagen (She) in mucosal protein extracts of insulin-treated rats and controls. The autoradiography showing the expression of the She subunits is depicted in Fig. 5, *top right*.
right, after 1 min of insulin stimulation anti-IRS-2 antibodies coimmunoprecipitated the two IRS (IRS-2 at \(\sim 185\) kDa and IRS-1 at \(\sim 165\) kDa), which were not tyrosine phosphorylated in control animals. Interestingly, IRS-1 and IRS-2 coimmunoprecipitated with another phosphotyrosine protein migrating at \(\sim 130\) kDa, which was not detected in controls. Because experiments were conducted under strong denaturing conditions, it is probable that p130 was detected as a protein directly associated with IRS-1 or IRS-2. Other members of the IRS family (i.e., IRS-4 and IRS-3) were unlikely candidates because of their molecular mass and relative mobility (25, 26). Indeed, IRS-3 migrates at 60 kDa (26) and IRS-4 at 160 kDa in SDS-PAGE (25). Figure 4, left, clearly shows the expression of IRS-4, immunoprecipitated with a specific anti IRS-4 antibody from weanling intestinal extracts, as a single insulin-elicited phosphotyrosine protein migrating at \(\sim 160\) kDa that was not phosphorylated in control rats. A likely candidate associated with IRS-2 in response to insulin could be PLC-\(\gamma\). Although PLC-\(\gamma\) was identified in intestinal protein extracts of weanling rats as a \(\sim 145\)-kDa phosphotyrosine protein (Fig. 4, middle), immunoprecipitation of IRS-2 followed by blotting membranes with anti-PLC-\(\gamma\) antibodies failed to confirm that the p130 associated with IRS-2 was PLC-\(\gamma\) (data not shown). Intestinal cells of weanling rats also expressed PKB, an important downstream substrate of PI 3-kinase, which binds to Glut-4-containing vesicles and is believed to activate p70/S6 kinase. The insulin dependence of PKB, aserine/threonine-phosphorylated kinase of 60 kDa, is shown in Fig. 4, right (relative abundance for insulin-treated and control rats was 6.29 ± 0.19 and 3.08 ± 0.10 OD units \(\times\) mm\(^2\), respectively).

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**Fig. 3.** Left: autoradiography of IRS-1 (p165) and a 185-kDa associated protein (presumably IRS-2) in mucosal protein extracts from weanling rats, treated with insulin intravenously (10\(^{-6}\) mol/l) for 1 min. After immunoprecipitation with anti-IRS-1 antibodies, these proteins were detected by Western blot, using antiphosphotyrosine antibodies (PY20). Both phosphotyrosine substrates were nearly undetectable in control animals. Right: autoradiography of IRS-2 (p185) and a 165-kDa associated protein (presumably IRS-1) in mucosal protein extracts from weanling rats, treated with insulin intravenously (10\(^{-6}\) mol/l for 1 min). After immunoprecipitation with anti-IRS-2 antibodies, these proteins were detected by Western blot using antiphosphotyrosine antibodies (PY20). Both substrates were not detected in control animals. Note the presence in insulin-treated-animals of a \(\sim 130\)-kDa associated protein whose nature remains unknown.

**Fig. 4.** Expression of IRS-4, phospholipase C-isoenzyme-\(\gamma\) (PLC-\(\gamma\)), and protein kinase B (PKB) in protein extracts from weanling rats (day 25), treated with insulin (10\(^{-6}\) mol/l iv for 3 min) or its vehicle. Left: intestinal proteins were immunoprecipitated (IP) with an anti-IRS-4 polyclonal antibody, and after electrotransfer, samples were immunoblotted (Western blot, WB) with antiphosphotyrosine antibodies (PY20). IRS-4 was detected by autoradiography as a single phosphotyrosine substrate of \(\sim 160\) kDa in insulin-treated rats but not in controls. Middle: PLC-\(\gamma\) was detected by the same method as a 145-kDa phosphotyrosine protein, present only in insulin-treated rats. Right: PKB was evidenced in insulin-treated rats as a single 60-kDa protein using a polyclonal anti-PKB antibody for both the immunoprecipitation and immunoblotting procedures (dilution 1:10,000). HC, heavy chain of the antibody.
The 85-kDa phosphotyrosine protein detected by probing protein extracts with antiphosphotyrosine antibodies (Fig. 1, left) was identified as the p85 subunit of PI 3-kinase, a heterodimer containing a regulatory subunit (p85) and a catalytic subunit (p110). Using a polyclonal antibody recognizing a large sequence (corresponding to amino acids 333–428 of the NH2-terminal SH2 domain) of human p85 subunit of PI 3-kinase, two isoforms, the α- and β-p85 subunits, were identified in the small intestine (Fig. 5, top left). The predominant isoform was α-p85. This adaptor molecule associates in other cell lines with the IR at tyrosine 1322 (44) and activates tyrosine kinase substrates (IRS-1, Grb2, SYP, and IRS-3) through SH2 domains and thus serves as a link between PI 3-kinase and other ligand-activated substrates (27, 35, 44, 55). The β-p85, a protein whose function remains unknown, was much less abundant. The same autoradiography shows a broad and intense signal extending from 50 to 55 kDa detected in both controls and insulin-treated rats. This suggests that, in addition to α-p85, two other isoforms, p55 and p50, previously evidenced in rat brain, muscle, and liver (19), are also expressed in the small intestine, especially because our antibody was raised against the entire NH2-SH2 domain of α-p85, which is common to the three isoforms α-p85, α-p55, and α-p50 (19). Figure 5, top left, also demonstrates a ~68-kDa protein that coimmunoprecipitated with the α- and β-p85, p55, and p50 isoforms of PI 3-kinase. Relative abundance of this protein (Fig. 2B) was greater in insulin-stimulated rats (3.84 ± 0.07 OD units × mm²) than in controls (2.43 ± 0.06 OD units × mm²). Its relative molecular mass was estimated by imaging densitometry to be 68 kDa in controls and 70 kDa in stimulated rats, the decrease in mobility likely being due to phosphorylation of residues in response to insulin. Attempts to identify the nature of this protein by probing immunoprecipitated p85 material with several antibodies against SH2 domain containing proteins remained unsuccessful. A potential candidate could be SYP (68 kDa), which contains two NH2-terminal SH2 domains interacting directly with the SH2 domain of p85 via its motif EY380GVM even under denaturing conditions (29, 55, 57).

Intestinal extracts from adult rats stimulated with insulin (10⁻⁶ mol/l for 3 min) and controls were immunoprecipitated and Western blotted with a polyclonal antibody reacting with a COOH-terminal peptide of the human Shc protein (amino acids 359–473 of the SH2 domain). This allowed the detection of three Shc proteins of ~66, 52, and 46 kDa, respectively (Fig. 5, top right). Densitometric measurements (Fig. 2C) showed that p46 was increased in relative abundance in response to insulin (insulin vs. controls, 3.16 ± 0.08 vs. 2.15 ± 0.03 OD units × mm², respectively), whereas p66 and p52 were equally abundant in insulin-treated and control rats, which could be explained by the presence of the heavy chain of the antibody.

The Shc family of proteins are believed to be important substrates of growth factor receptors, including growth receptor-bound protein-2 (Grb2, p24) in intestinal extracts from rats treated with insulin (10⁻⁶ mol/l for 3 min) or its vehicle. The p55 and p50 subunits form a large complex that was not separated by the electrophoretic conditions. Protein extracts were immunoprecipitated with a polyclonal antibody raised against the p85 subunit of PI 3-kinase, and the immunoprecipitated proteins were detected with the same antibody in Western blot. Note that the PI 3-kinase subunits coimmunoprecipitated with a 68-kDa protein, whose abundance was increased in insulin-treated rats. Although the nature of this protein is unknown, it could presumably be the protein tyrosine phosphatase-2. Top right: expression of the 3 subunits (p66, p52, p46) of Shc in mucosal extracts prepared from insulin-treated rats (10⁻⁶ mol/l for 3 min) and controls immunoprecipitated with anti-Shc antibodies. Note that abundance of the p46 subunit is enhanced in insulin-treated rats. Immunoprecipitated proteins were detected with the same antibody in Western blot. Bottom middle: expression of the growth receptor-bound protein-2 (Grb2, p24) in intestinal extracts from rats treated with insulin (10⁻⁶ mol/l for 3 min) or its vehicle. Grb2 was probed directly with anti-Grb2 antibodies on polyvinylidene difluoride membranes. The protein was clearly present in insulin-treated rats but not in controls. LC, the end of the gel.

### Table 1. Activity of protein kinases in immunoprecipitated mucosal samples from insulin-treated rats and controls

<table>
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<th>Kinase</th>
<th>Controls</th>
<th>Treated</th>
<th>Fold Increase</th>
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<td>PKB</td>
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<td>p70/S6 kinase</td>
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Values are means ± SE given in pmol/substrate phosphorylated-min⁻¹-g mucosa⁻¹; n = 3–7 individual determinations, each sample corresponding to a pool of intestinal mucosa from 2 rats. PKB, protein kinase B; MAP kinase-2, mitogen-activated protein kinase-2; p70/S6 kinase, p70 ribosomal S6 kinase.
the IR, and associate with Grb2/sem5 gene products that link receptor tyrosine kinases and p21 Ras, suggesting a role for Shc in the Ras-GAP pathway (17, 20).

We also identified in intestinal extracts of insulin-treated rats Grb2 as a ~24-kDa tyrosine phosphorylated protein, which was undetectable in control rats (Fig. 5, bottom middle). Grb2 is a small cytoplasmic protein containing two SH3 domains and one SH2 domain (52), which interacts in response to IR activation with mammalian son of sevenless (mSOS), IRS-1, and Shc via its SH2 domain (17, 36). Like in target cell lines stimulated in vitro with insulin or with other growth factors (epidermal growth factor, platelet-derived growth factor), we have detected in intestinal extracts from weanling rats (day 23) a ~120-kDa protein identified as GAP (the mean GTPase-activating protein of the normal form of p21 Ras). In animals injected with insulin (10^{-6} mol/l, 1 min) immunoprecipitation and Western blotting of protein extracts with an anti-GAP polyclonal antibody, reacting with a large protein fragment of human GAP (corresponding to amino acids 171–443, which include 2 adjacent SH2 domains and 1 SH3 domain), revealed a major signal of ~120 kDa (p120 GAP) and two phosphorysensitive proteins of ~190 (Rho GAP) and ~62 kDa (p62Src), respectively (Fig. 6, left). The p120 GAP was also present in control rats (relative abundance: 4.07 ± 0.20 OD units x mm^{2}), but its abundance was stimulated threefold (n = 4) in insulin-treated rats (11.59 ± 0.31 OD units x mm^{2}). Interestingly, p190 Rho GAP and p62Src protein were not detected in controls but coimmunoprecipitated with p120-GAP only in response to insulin, indicating that their interactions with p120 GAP are physiologically relevant and likely representing a direct binding between these proteins. Likewise, in cell lines stimulated in vitro with growth factors, Ras-GAP is phosphorylated on both tyrosine and serine residues and forms complexes with two phosphorylated proteins, p190 Rho GAP and p62Src. These associations inhibit the GTPase activity of Ras-GAP (44, 55).

MAP kinases (extracellular signal-activated kinases or ERK) are cytoplasmic and nuclear protein kinases acting as intermediates between the insulin-stimulated phosphorylated cascade (Ras-GAP pathway) and the response of eukaryotic cells to extracellular signals (32). To identify MAP kinase-1 and -2 in rat small intestine, we immunoprecipitated protein extracts from adult rats stimulated with insulin (10^{-6} mol/l for 3 min) or saline because MAP kinase expression increases during development (4). The polyclonal antibody used was raised against a 36-amino-acid peptide (PFTFMDFLDLPKSERLKLIFQETARFQPGAPEAP) corresponding to the COOH terminus of rat MAP kinase-1. As shown in Fig. 6, right, both MAP kinase-1 (p44) and MAP kinase-2 (p42) were tyrosine phosphorylated in response to insulin and were not detected in control rats. The antibody used also recognized MAP kinase-2 because the rat immunogenic sequence of MAP kinase-1 has 90% identity with mouse or human MAP kinase-2 (BLAST search). The corresponding signals shown in Fig. 6, right, are not abundant after probing the immunoprecipitates with antiphosphotyrosine antibodies (PY20), because these proteins are serine/threonine kinases (4, 32) and are phosphorylated in response to insulin only on one tyrosine site (Tyr^{205}) and one threonine site (Thr^{203}) (4, 32). In concordance, the activity of MAP kinase-2 measured in immunoprecipitated samples was two times higher (P < 0.01, n = 7) in insulin-treated rats than in controls (n = 5) (Table 1).

Response of rat immature intestinal mucosa to in vivo administration of inhibitors. The effects of the IP administration of a monoclonal anti-IR antibody (RPN 538) recognizing epitopes of the α-extracellular subunit on the specific activity of BBM hydrolases are detailed in Table 2. Sucklings treated with two doses of 20

![Fig. 6. Left: mucosal protein extracts of weanling rats (day 25) untreated or treated with insulin (10^{-6} mol/l, 3 min) were immunoprecipitated with anti-GTPase-activating protein (GAP) antibodies. The immunoprecipitated proteins were detected by Western blot using the same antibody. The resultant autoradiography shows 3 proteins, p190 (Rho-GAP), p120 (GAP), and p62 GAP-associated protein, whose abundance was markedly enhanced in insulin-stimulated animals. In controls the relative abundance of GAP (p120) was decreased while Rho-GAP and p62Src-GAP-associated protein were nearly absent. Because of a slight deviation in the run, the figure of the autoradiography has been cut between 62 and 120 kDa to perfectly align the signals in the lanes of insulin-treated rats and controls. Right: expression of mitogen-activated protein kinases (MAP kinases) in mucosal extracts of adult rats treated with insulin (10^{-6} M for 3 min) or with its vehicle. MAP kinase-1 (p44) and -2 (p42) were immunoprecipitated using a polyclonal anti-MAP kinase-1 antibody and detected by Western blot using antiphosphotyrosine antibodies (PY20). Both kinases were not detected in control animals.](http://ajpgi.physiology.org/ by 10.220.33.5 on October 29, 2017)
μg/day of the antibody from day 11 to day 14 postpartum had growth rates similar to saline controls. However, mucosal weight expressed per centimeter of gut length was significantly lower in the monoclonal-treated group with depression by day 14 of the specific activity of SI and maltase. There was no change in the activity of lactase between the two groups. Corticosterone was equivalent between the three groups of rats (mean: 1.34 μg/dl, saline controls; 1.54 μg/dl, anti-IR treated group; and 1.39 μg/dl, insulin-treated group). These hormone levels are negligible compared with the active circulating levels measured in 18-day-old weanling rats (17.6 μg/dl) (7, 9). The identification in rat small intestine of the regulatory subunits (α-p85, β-p85, α-p55, and α-p50) of PI 3-kinase by immunoprecipitation and Western blot prompted us to determine whether this key enzyme could regulate a specific pathway stimulating BBM enzymes. Wortmannin, an irreversible inhibitor of PI 3-kinase, was administered at low doses of 5 μg/g body wt (median lethal dose = 500 μg/g) to sucklings for 72 h, 1 h before the administration of insulin. The results are presented in Table 3. Growth rates were similar in the wortmannin-treated group and controls. There was no change in BBM protein concentration or in mucosal mass expressed per unit of length. Surprisingly, compared with insulin-treated controls, wortmannin enhanced SI activity fivefold, maltase activity 2.7-fold, and lactase activity 1.7-fold. Measurements of plasma insulin levels revealed a marked increase in insulinemia in the wortmannin-treated group (wortmannin vs. controls, 67 ± 0.5 vs. 10 ± 0.1 μU/ml, respectively).

Table 3. Response of rat immature small intestine to insulin alone or wortmannin and insulin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Insulin Controls</th>
<th>Wortmannin + Insulin</th>
<th>Fold Increase</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrase, μU/mg BBM protein</td>
<td>8.4 ± 4.0</td>
<td>41.5 ± 14.1</td>
<td>5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Maltase, μU/mg BBM protein</td>
<td>534 ± 173</td>
<td>1,447 ± 315</td>
<td>2.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lactase, μU/mg BBM protein</td>
<td>252 ± 35</td>
<td>434 ± 33</td>
<td>1.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mucosal wt, mg/cm</td>
<td>3.07 ± 0.2</td>
<td>3.05 ± 0.1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Glycemia, mg/dl</td>
<td>87 ± 7.0</td>
<td>93 ± 2.1</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats. Insulin and wortmannin + insulin were administered on days 11–14 postpartum.

Because MAP-kinases I and II are known to be critical enzymes regulating the mitogenic effects of insulin, PD-98059, a specific inhibitor of MAP kinase, was administered intraperitoneally to sucklings at low doses (2 μg/g body wt twice daily) (median lethal dose = 200 μg/g) for 48 h, 1 h before the administration of insulin. As detailed in Table 4, sucklings treated with PD-98059 and insulin gained weight as did the control group without change in final intestinal length. However, mucosal mass expressed per centimeter of length was significantly lower in the experimental group compared with the control group. Sucrase activity was 3.7 times lower in the experimental group than in the insulin-treated group, suggesting that PD-98059, by inhibiting MAP kinases, prevented the ontogenic induction of the enzyme. In concordance, maltase and lactase activities decreased 42% and 44%, respectively, compared with the activities measured in controls.

DISCUSSION

Our studies had two complementary goals: first to identify in the intestinal cells of intact living animals, under physiological conditions, the receptor substrates and phosphorylated proteins involved in the signal transmission of insulin and second to approach the signal pathway activating BBM hydrolases. To accomplish these objectives, we adapted to the small intestinal mucosa the method of Rothenberg et al. (41) used to

Table 4. Response of rat immature small intestine to MAP kinase inhibitor PD-98059

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Insulin Controls</th>
<th>PD-98059 ± Insulin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (day 12)</td>
<td>17.6 ± 0.79</td>
<td>18.1 ± 0.66</td>
<td>NS</td>
</tr>
<tr>
<td>Final (day 14)</td>
<td>19.3 ± 0.82</td>
<td>21.0 ± 0.67</td>
<td>NS</td>
</tr>
<tr>
<td>Intestinal length, cm</td>
<td>40.1 ± 1.32</td>
<td>41.0 ± 1.79</td>
<td>NS</td>
</tr>
<tr>
<td>Mucosal wt, mg/cm</td>
<td>8.73 ± 1.32</td>
<td>7.28 ± 0.38</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sucrase, μU/mg BBM protein</td>
<td>2.10 ± 0.23</td>
<td>0.56 ± 0.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Maltase, μU/mg BBM protein</td>
<td>368 ± 32</td>
<td>252 ± 22</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lactase, μU/mg BBM protein</td>
<td>258 ± 35</td>
<td>171 ± 16</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BBM protein, mg/ml</td>
<td>5.32 ± 0.76</td>
<td>4.75 ± 0.63</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats. PD-98059 was administered twice daily on days 12–14 postpartum.
extract phosphotyrosine proteins from rat liver. Our results demonstrate that the SDS denaturation method allows a direct assessment in vivo of insulin-elicited tyrosine phosphorylation of endogenous substrates of physiological significance. There is so far no information regarding the mechanism(s) by which the signal of insulin is transduced from the IR downstream into the intestinal cell. The main reason is probably that intestinal cells are not considered to be typical target cells for insulin, although the hormone is essential for intestinal growth and cell maturation. Using the denaturing method of protein extraction, coupled with immunoprecipitation and immunoblotting proteins with antiphosphotyrosine antibodies, two proteins migrating at ~165 and 94 kDa (corresponding to IRS-1 and the β-subunit of the IR) were tyrosine phosphorylated in the small intestine of insulin-treated rats but not in controls (Fig. 1, right). A major complex of several phosphotyrosine proteins ranging from ~55 to 68 kDa was also immunoprecipitated and detected without difference between insulin-stimulated and control rats. However, when immunoblots of protein extracts were probed directly with antiphosphotyrosine antibodies (PY20), we detected in insulin-treated rats at least nine individual phosphotyrosine spots (p185) in intestinal cells of insulin-stimulated rats and can activate PI 3-kinase (47). Furthermore, two other IRS candidates have been sequenced: IRS-3 in adipocytes (26) and IRS-4 in human embryonic kidney cells (25). In the present study, IRS-4 was also detected in rat intestinal cells. In contrast to IRS-2, IRS-4 was still phosphorylated after 3 min of insulin stimulation. The aligned structure of all of the members of the IRS family is relatively similar and includes from the NH₂ terminus a highly conserved pleckstrin homology domain (PH domain), followed by a highly conserved protein tyrosine-binding domain (PTB domain), a non-PTB domain referred to as the S1AIN domain (IRS-1 and IRS-2), and a second COOH-terminal domain (IRS-2) containing multiple tyrosine phosphorylation sites that can bind to various SH2 proteins (13, 25). Although the PH and PTB domains are highly conserved in all IRS, there is little homology between the COOH-terminal domains in IRS-1 and IRS-2 and the corresponding regions in IRS-3 and in IRS-4 (25, 26). Binding specificity to SH2 proteins is determined by the amino acid sequence motif around the phosphotyrosine residue (52, 57). For instance, upstream both the IRS-1 and IRS-2 domains of IRS-2 interact with phosphorylated NPXY motifs in the receptors for insulin, IGF-1, and interleukin-4 (12, 16), whereas downstream IRS-1 interacts with at least four sites of the SH2 domains of α- and β-p85: Y⁶⁸⁸MPP, Y⁹³⁹MPP, Y⁹⁸⁷MTM, and Y⁵⁶⁹ICM (45). PI 3-kinase plays a pivotal role in signal transduction. In this study in the rat small intestine, we have detected four isoforms of the regulatory subunits of PI 3-kinase: α- and β-p85, p55, and p50 (fused). Similar findings have been published by Inukai et al. (19) in the rat liver. Interestingly, p50 exhibited higher capacity for activation of associated PI 3-kinase via insulin stimulation and had a higher affinity for tyrosine-phosphorylated IRS-1 than the other isoforms (19). Each isoform has a different tissue distribution and may have specific functions in various tissues. The regulatory subunits (p85) of PI 3-kinase activate the serine/threonine kinase PKB, Grb2, mSOS, p21 Ras-GAP, the MAP kinase cascade, and finally the nuclear translocation of a family of cytoplasmic transcription factors called STATS (for signal transducer and activator of transcription) (43). This cascade of activations results in the promotion of glucose uptake, glycogen synthesis, mitogenesis, or gene expression according to the specificity of the target tissue.
by insulin, whereas the basal activity of p70/S6 kinase remained unresponsive to the hormone.

Besides the phosphorylation of IRS-1 and IRS-2, activation of the IR phosphorylates another cellular substrate, Shc. As shown in Fig. 4, Shc is expressed in the rat small intestine as three proteins, p46, p52, and p66, which differed in relative abundance. Each Shc protein contains a NH$_2$-terminal PTB domain, a central glycine/proline rich sequence, and a COOH-terminal SH2 domain (17, 20, 56). The three Shc proteins are phosphorylated by activated growth factor receptors (e.g., insulin, EGF, IGF-1) and oncogen tyrosine kinases to form complexes with Grb2. We also clearly demonstrated in intestinal protein extracts of insulin-stimulated rats an increase in abundance of the p120-Ras-GAP and the presence of two associated proteins, p190 (Rho GAP) and p62$^{src}$. Rho GAP is a phosphotyrosine protein tightly bound to p120 GAP in nearly stoichiometric amounts. The virtual absence of RhoGAP and p62$^{src}$ in intestinal extracts from control rats indicates that the associations between these molecules and p120 GAP likely represent a direct binding. This is further attested to by the fact that Rho GAP exhibits three domains that share strong homology with GTP-binding proteins, (Rho) GAP-like molecules, and the putative glucocorticoid gene repressor (42). When the cell is stimulated, Rho-GAP appears to be an effector acting via GAP to transduce signals from p21 Ras to the nucleus, because ~25% of the immunoprecipitated p190 is detected in the nuclear compartment (42). The p62$^{src}$ GAP-associated protein could be another effector of the p21 Ras pathway, because it contains DNA- and RNA-binding domains and exhibits similarities to nuclear ribonucleoproteins (38, 53).

The mechanism(s) by which insulin stimulates enzyme expression in rat immature enterocytes has been little investigated. Besides receptor activation, a direct effect of insulin on target cells, unrelated to the receptor status, has been postulated. Experimental evidence (33) has shown that insulin can exert mitogenic effects in Xenopus laevis oocytes by direct contact with the nuclei in the absence of membrane receptors. These observations prompted us to clarify whether IR binding is necessary for inducing BBM enzyme expression. The monoclonal antibody used here recognizes extracellular epitopes of the α-subunit of the human IR, whose molecular structure closely resembles that of the rat. When administered at low doses to suckling pups, anti-IR antibodies clearly prevented the action of endogenous insulin by inhibiting mucosal growth and the ontogenic expression of sucrase and maltase compared with age-matched controls. These data are in agreement with our previous study on insulin B-ASP$^{15}$ (11) receptor binding and provide unequivocal evidence that endogenous insulin plays a physiological role in the ontogenic expression of BBM enzymes. As a result, the downstream signal is at least in part triggered by binding of the hormone to the extramembranous sites of the IR. Similar effects of anti-IR antibodies on the growth of chicken embryos have been observed by Girbau et al. (15). In the study (15), it was found that administration of 200 and 400 μg protein/day resulted in a dose-related inhibition of growth with marked depression of total body DNA, RNA, and protein contents of the embryos, resulting in a mortality of 20–40%, proportional to the dose of antibody given. The inhibition of PI 3-kinase by its irreversible inhibitor wortmannin not only failed to block the enzymatic response to insulin but significantly stimulated the BBM enzyme activities by an overproduction of insulin. This insulin overproduction was apparently induced by wortmannin itself, because the control and experimental groups received the same doses of insulin. A similar observation has recently been published by Nuno$^{11}$ et al. (37). Perfusion of freshly isolated rat pancreas islets by wortmannin (10$^{-4}$ to 10$^{-8}$ M) markedly enhanced insulin secretion by the inhibition of phosphodiesterase, resulting in increased cAMP content. Despite inhibition of PI 3-kinase, the enzymatic response to insulin was not inhibited, suggesting that PI 3-kinase is not critical for the transduction of the signal.

The detection of MAP kinase-1 and -2 in the rat small intestine and the stimulation of their activity by insulin (Fig. 6) prompted us to inhibit their activation, especially because these key enzymes downregulate the mitogenic effects of insulin. PD-98059 is a specific inhibitor of MAP kinase kinase that in turn activates MAP kinase-1 and -2. The administration of PD-98059 to suckling pups at low doses clearly inhibited both mucosal mass and the expression of BBM hydrolases after 48 h of treatment (Table 4). Together, these results suggest that after receptor binding the signal of insulin that produces mitogenic effects and BBM enzyme stimulation is transduced via the pathway (Grb2, SOS, Raf, Ras-GAP?) that activates MAP kinase-1 and -2. Because our (11) previous studies have shown that the stimulation of gene transcription by insulin is independent of the mitogenic effects of the hormone, further studies are warranted to clarify the role of this signaling pathway in the expression of BBM enzymes.

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