The function of mitogen activated protein kinases in zebrafish development

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Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines

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Abstract

Chitin oligosaccharides (COs) play a role in plant development and are presumed to affect body plan formation during vertebrate embryogenesis. The mechanisms of COs recognition and cellular processes underlying embryonic development are still not understood. We analyze the possible link with the mitogen-activated protein kinase pathway that is conserved in evolution through the plant and animal kingdom and has been implicated in diverse cellular processes, including cell growth, proliferation, differentiation, survival, and vertebrate development.

We show that *in vivo* stimulation of embryonic zebrafish cells ZF13 and ZF29 with chitin tetrasaccharides at 10^{-9} M concentration transiently induced activation/phosphorylation of extracellular regulated kinases (ERKs), with a maximum after 15 minutes. Furthermore the biological specificity of chitin tetrasaccharides and various derivatives was examined. The replacement of one or two GlcNAc residues of the chitin backbone by glucose and fucosylation of chitin tetrasaccharides at the reducing terminus caused a complete loss of their activity. We also tested a chitin tetrasaccharide analogue in which the oxygen atoms in glycosidic linkages were replaced by sulfur atoms. This analog, which could not be enzymatically hydrolyzed, was as potent an inducer as chitin tetrasaccharide. These results suggest that the observed activation of ERKs is chitin tetrasaccharide-specific and does not require further enzymatic processing. We examined possible signaling pathways leading to ERK activation by COs by use of phosphospecific antibodies and inhibitors. We conclude that a high-affinity CO receptor system exists that links to the Raf, MEK, and ERK pathway in zebrafish cells.

Introduction

Chitin, a repeating $\beta(1\rightarrow4)$-linked homopolymer of the monosaccharide N-acetylglucosamine (GlcNAc), is one of the most widespread and abundant molecules in the biosphere (Varki, 1996). Chitin oligosaccharide (CO) derivatives play a role in the leguminous plant development and possibly also in vertebrate embryogenesis (Bakkers et al., 1997). Rhizobial NodC protein, responsible for CO synthesis, is homologous to the developmentally regulated DG42/HAS protein of vertebrates (Bulawa and Wasco, 1991; Semino and Robbins, 1995). Members of the DG42/HAS family have been shown to be involved in the biosynthesis of both hyaluronan and COs (DeAngelis and Acbyuthan, 1996; Meyer and Kreil, 1996; Semino et al., 1996; Yoshida et al., 2000; Van der

Holst et al., 2001). A possible biological function for COs was elucidated by microinjection studies of fertilized zebrafish eggs with anti-DG42 antiserum and the rhizobial NodZ fucosyltransferase (Bakkers et al., 1997). These treatments led to severe defects in trunk and tail development, suggesting that COs may function as signaling molecules in cell growth, differentiation, and development of vertebrates (Semino et al., 1998; Semino and Allende, 2000).

Mammalian cells respond to a variety of extracellular stimuli via activation of specific mitogen-activated protein kinases (MAPKs) that orchestrate the transduction of the signal from receptors at the cell surface to the nucleus and play a major role in the integration of multiple biological responses. Three main classes ofMAPK are recognized: the classic MAPK, also known as extracellular-regulated kinases, ERK1 and ERK2, and c-Jun N-terminal kinase (JNK) and p38 MAPK, which are activated by dual phosphorylation at neighboring threonine and tyrosine residues in the activation loop. Dephosphorylation of either residue results in MAPK inactivation (Johnson and Lapadat, 2002). The general scheme of ERK activation involves a cascade of phosphorylation events initiated by stimulation of the Ras proto-oncogene following activation of growth factor receptors. The cascade starts with the activation of one or more Raf family kinases, which phosphorylate and activate the MAPK kinases (MEK1/2). MEK in turn catalyzes dual phosphorylation and activation of ERKs (Chang and Karin, 2001). Once activated, ERKs accumulate in the nucleus by as-yet undefined mechanisms that seem to involve the dissociation of ERK from MEK (Fukuda et al., 1997) and/or a blockade of its export from the nucleus by neosynthesized nuclear anchors (Lenormand et al., 1998).

The ERK pathway has been implicated in diverse cellular processes, including cell growth, proliferation, differentiation,and survival (Marshall, 1995; Ballif and Blenis, 2001). Hence, finely tuned regulation of ERK activation is essential in conveying appropriate signals. The intensity, duration, and subcellular localization of ERK activation are well regulated. Scaffolding proteins and docking sites provide the means to avoid cross-activation between MAPK signaling pathways and permit precise and even cell-specific subcellular localization of ERKs (Pouysségur et al., 2002). The variable responses elicited by this cascade in different cell types are also presumably determined by the cellspecific combination of downstream substrates.

Activated ERK phosphorylates numerous substrates in all cellular compartments (Lewis et al., 1998). More than 50 different ERK substrates have been identified so far. These include ubiquitous or lineage-restricted transcribed factors, the kinases RSK and MNK and proteins involved innucleotide biosynthesis, cytoskeleton organization, ribosomal transcription, and membrane traffic (Sturgill et al., 1988; Marais et al., 1993; Treisman, 1996; Fukunaga

and Hunter, 1997; Lewis et al., 2000; Stefanovsky et al., 2001). RSKs, 90-kDa ribosomal S6 serine/threonine kinases family (also known as MAPK-activated protein kinase-1,MAPKAP-K1) are activated by ERKs *in vitro* and *in vivo* via phosphorylation (Sturgill et al., 1988; Dalby et al., 1998).

The presence and function of MAPK pathways in zebrafish has been recently described. The insulin-like growth factor stimulates zebrafish cell proliferation by activating MAPK and PI3-kinase-signaling pathways (Pozios et al., 2001), while FGF/MAPK signaling is required for development of the subpallial telencephalon and somite boundary formation in zebrafish embryos (Shinya et al., 2001; Sawada et al., 2001). Hence components upstream and downstream of the specific MAPK cascades in zebrafishare not known yet.

Here we show that chitin tetrasaccharides rapidly and specifically activate the ERK pathway in embryonic zebrafish cells. These findings provide strong evidence that CO signaling is initiated on the plasma membrane via activation of high-affinity oligosaccharide receptor system, whichtransmits the signal to nucleus probably using the Ras, Raf, MEK, ERK cascade.

Results

Activation of ERK by chitin tetrasaccharides

To investigate a possible role of COs as signaling molecules in vertebrate development, the embryonic zebrafish cell lines ZF13 and ZF29 were used as a model (Peppelenbosch et al., 1995) and the canonical MAPK pathway was selected as a well-characterized junction between extracellular signals and integrated biological responses. EGF was used as a control of functionality in the system.

Zebrafish cells were serum starved for 24 h, stimulated with $5x10^{-6}$ M chitin tetrasaccharides and EGF for the indicated times, lysed, and processed for western blotting. The activated/phosphorylated form of MAPKs, ERKs was monitored by specific dual phospho-p44/42 MAP kinase antibodies (dp ERK), which detect p44 and p42 MAP kinase (ERK1 and ERK2) only when catalytically activated by phosphorlation at Thr202 and Tyr204. The antibody does not appreciably cross-react with the corresponding phosphorylated threonine and tyrosine residues of either JNK/SAPK or p38 MAPK homologs. It does not cross-react with up to 2 mg non-phosphorylated p44/42 MAPK. The total protein level in all performed experiments was control by ERK1 antibody, which reacts with ERK1 (p44) and to a lesser extent ERK2 (p42), by western blotting. Bands intensities were quantified by densitometry, and ERK activity was com-

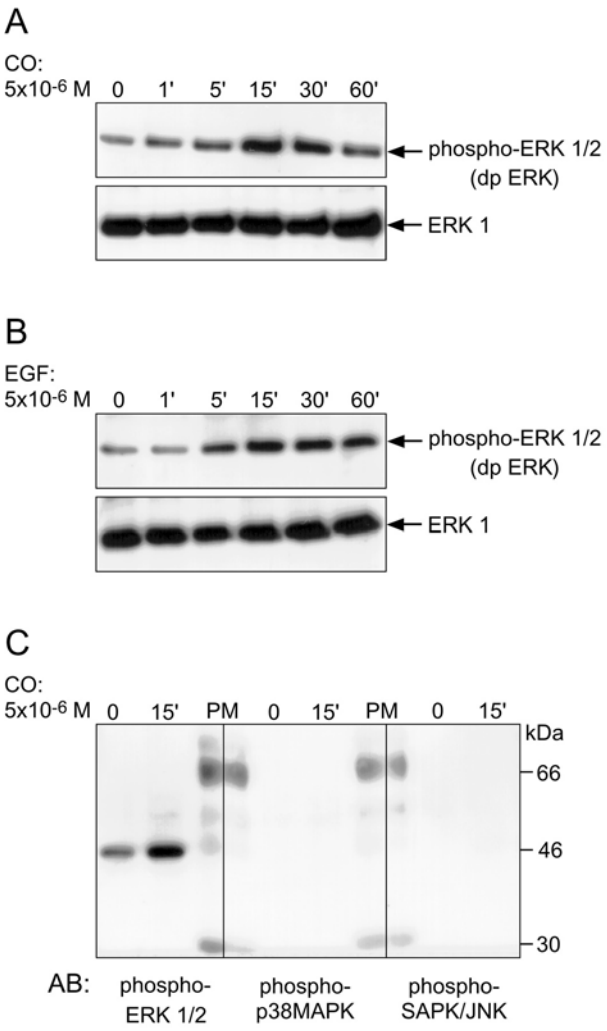

Figure 1. Time-dependent and specific activation of ERKs by COs and EGF. Serum-starved zebrafish cells (ZF13) were stimulated with $5x10^{-6}$ M COs (A) and EGF (B) for increasing periods of time (0-60 min). ERK activation were monitored by immunoblotting of total cell lysates with specific phospho-MAPK (Thr202/Tyr204) antibody (dpERK). The total amount of ERK was visualized using an ERK1 antibody. These results are representative examples of five experiments and were identical for ZF29 cells (data not shown). The lysates obtained from not stimulated (0) and 15 min stimulated (15') cells with $5x10^{-6}$ M CO and protein marker (PM) were subjected to western blotting. The specificity of ERK phosphorylation was determined with anti-phospho-ERK1/2, anti-phospho-p38 MAPK, and phospho-SAPK/JNK antibodies (C).

pared to the basal level at t=0.

In vivo stimulation of ZF13 and ZF29 with chitin tetrasaccharide and EGF transiently induced the 8-15 fold activation/phosphorylation of ERKs, with a maximum after 15 min (Fig.1). The half maximal response was obtained at 10^{-9} M concentration (Fig.3). Although dp ERK antibodies are able to recognize both phosphorylated ERK1 and ERK2 in all experiments, one band of 44 kDa was preferentially detected, suggesting that ERK1 is mainly activated by COs and EGF. The anti-phospho-p38 MAPK and anti-phospho-SAPK/JNK antibodies showed absence of cross-reaction in the CO activation process observed with phospho-ERK1/2 antibody, ensuring the specificity in the western blots (Fig.1C).

ERK activation is specific for chitin tetrasaccharides, CO, and thio-CO

We examined the biological specificity of chitin tetrasaccharides and various derivatives (Fig.2). The replacement of one or two GlcNAc residues of the chitin backbone by glucose and fucosylation of chitin tetrasaccharides by NodZ protein abolished the biological activity of CO(2glc)-MP and CO-fuc in the tested concentration of 10^{-7} M (Fig.3A). This loss of activity was apparently not due to modifications at C1 position of the chitin tetrasaccharide molecule because para-nitrophenyl chitin (CO-PNP) that is structurally very similar to paramethoxy- phenyl (MP) modified at this position showed normal activation of ERK (Fig.2 and 3A). The C2 and C3 position of CO molecule seem to be essential for its biological property. We also tested a chitin tetrasaccharide analog, thioCO, in which the oxygen atoms in glycosidic linkages were replaced by sulfur atoms. This analog, which cannot be enzymatically hydrolyzed (Wang and Lee, 1995) was as potent an inducer as chitin tetrasaccharide. The half-maximal responses, induced by 15 min treatment with CO and thioCO, were obtained at 10^{-9} M concentration for both compounds (Fig.3A). These results suggest that the observed activation of ERKs is chitin tetrasaccharide± specific and does not require further enzymatic processing.

Signaling pathway to ERK activation by chitin tetrasaccharides

In general, ERK activation involves a cascade of phosphorylation events initated by stimulation of the Ras protooncogene following activation of growth factors receptors. The cascade starts with the activation of Raf, which phosphorylates MAPK kinase (MEK) on two serine residues, and then ERK is dually phosphorylated on a tyrosine and threonine residues by MEK. Activated ERK phosphorylates specific serines and threonines of target protein substrates.

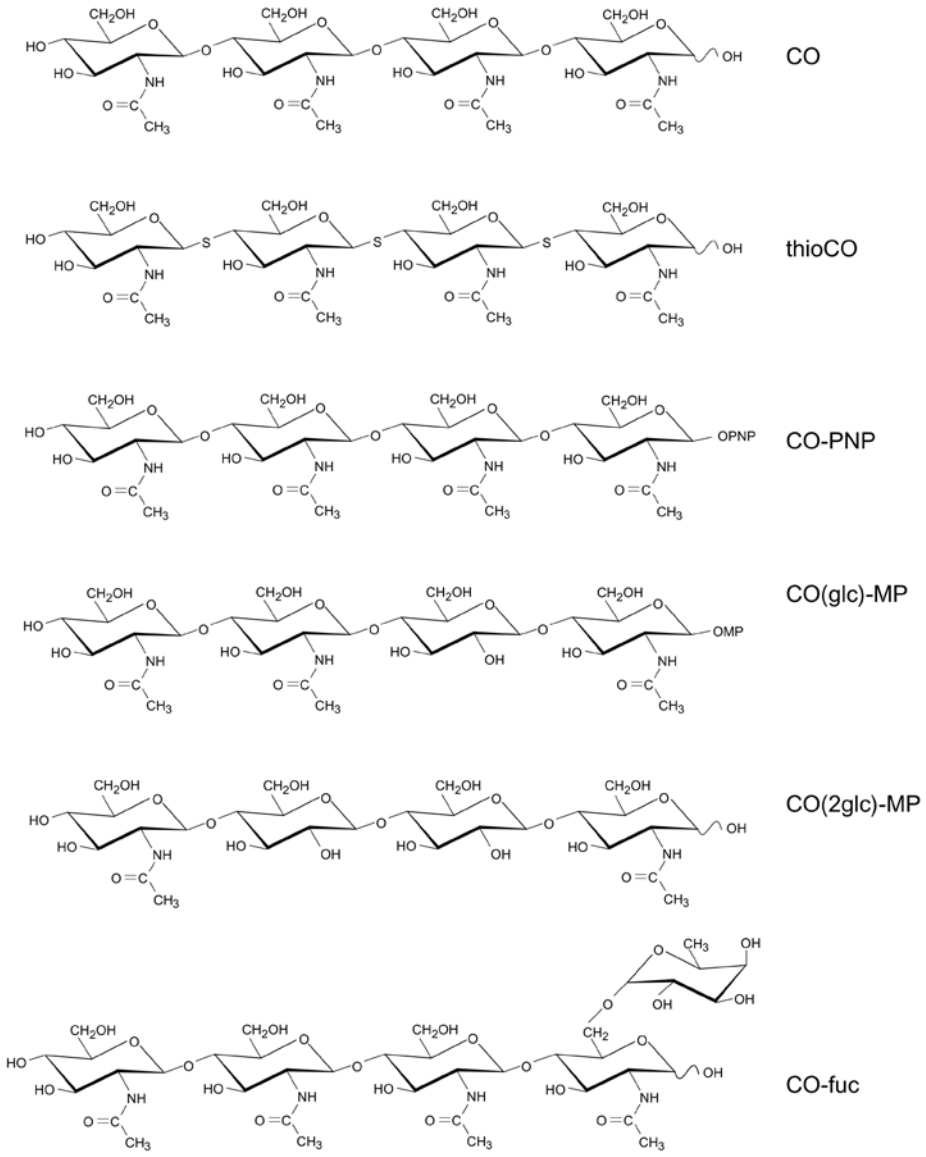

Figure 2. Structures of CO derivatives. CO: chitin oligosaccharide; thioCO: CO in which the oxygen atoms of the β(1→4) linkages are substituted by sulfur atoms; CO-PNP: CO with PNP modification at C1 position; CO(Glc)-MP and CO(2Glc)-MP: one or two GlcNAc residues are replaced by glucose, respectively, and with MP modification at C1 position; CO-fuc: fucosylated CO.

Several downstream targets of ERK1/2 have been identified, for instance, p90RSK is activated by ERK1/2 via phosphorylation at Ser380 (Dalby et al., 1998). To examine a possible signaling pathway to ERKs activation by chitin tetrasaccharides, we made use of phospho-Raf (Ser259), phospho-MEK1/2 (ser217/221), and phospho-p90RSK (Ser380) antibodies to follow expected phosphorylation events in ERK activation cascade. The activation of MEK1/2 and subsequently ERK were blocked by a highly selective inhibitor of MEK1 and MEK2, UO126, at 10^{-5} M concentration (Crews et al., 1992). The result in figure 4A show that CO, thioCO, and EGF induced phosphorylation/ activation of Raf, MEK1/2, and p90RSK with the same kinetics and specificity observed for ERK activation. Again CO-fuc and CO(2glc)-MP were found to be not active (Fig.3A and 4A). Preincubation of cells for 1 h with MEK1/2 inhibitor before stimulation prevented stimulation of ERK phosphorylation by CO and EGF (Fig.4B). From this we conclude that chitin oligosaccharides are recognized by a high-affinity receptor system that activates Raf, MEK, and ERK pathway in these zebrafish cell lines.

Discussion

Many human diseases, including the formation of tumors and their subsequent metastatic spread through the body, are caused by defects in carbohydrates recognition (Schuster and Nelson, 2000). Furthermore, all human pathogens also naturally produce a great variety of carbohydrates and use an unknown number of these molecules to modulate the innate immune response of the host (Aderem and Ulevitch, 2000). Most of these carbohydrates are polymers either in free form or linked to proteins. However, evidence exists that also small oligosaccharides, such as COs, play a crucial role in animal development. The genetic and cellular basis of the CO recognition mechanisms underlying their function in embryonic development, tumorigenesis, or infectious diseases is still not understood. The zebrafish model system offers novel opportunities to study the interplay between signal perception, transmission, and development. In this study we address whether COs are able to function as signaling molecules to activate the canonical ERK pathway in embryonic zebrafish cell cultures. The MAPK/ERK pathway was chosen as biological tool in this project because it plays a major role in the integration of multiple biological responses controlling development (Pouysségur et al., 2002). The activation of ERKs and downstream components was monitored by phosphospecific antibodies because the general scheme of activation involves a cascade of phosphorylation events following activation of specific receptors

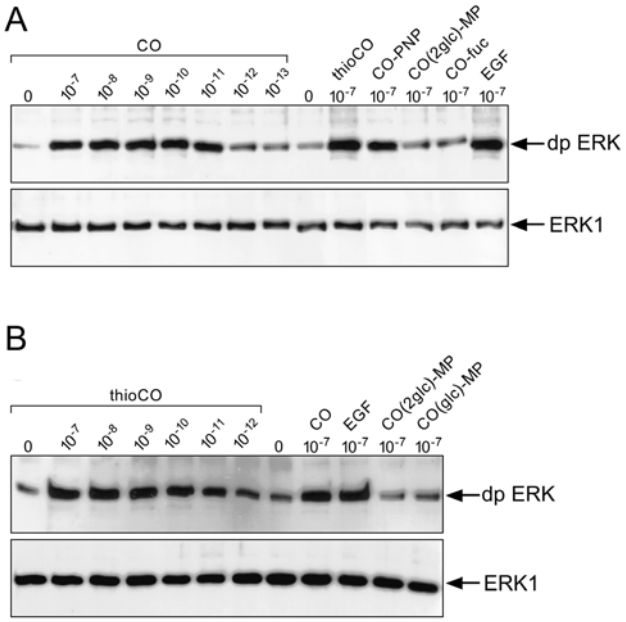

Figure 3. Dose dependence and specificity of ERK activation by CO and derivatives. Serum-starved zebrafish cells (ZF13) were stimulated with various concentrations of CO, CO(2glc)-MP or CO-fuc (A) and thioCO (B) for 15 min. Phosphorylated and nonphosphorylated forms of ERK were detected by dp ERK and ERK1 antibodies. These results are representative of three experiments. 10^{-7} M EGF was used as a control in A and B.

by appropriate signal.

First, we found that chitin tetrasaccharides transiently induced phosphorylation of ERKs, probably ERK1, with a maximum after 15 min stimulation, at 10^{-9} M concentration. This response was very specific for the structure of the glycan backbone. The replacement of one or two GlcNac residues by glucose and fucosylation by the NodZ protein resulted in a loss of their biological activity. In contrast, an analog of CO, thioCO, which could not be enzymatically hydrolyzed, was as potent an inducer as COs, excluding a role of possible degradation products. Furthermore, we also examined the signaling pathway to ERK activation by COs and concluded that a high-affinity oligosaccharide receptor system exists that transduces the signal to Raf, MEK, and ERK. Once activated/phosphorylated ERK phosphorylates p90RSK kinase, known as MAPK-activated protein kinase-1, MAPKAP-K1. Phosphorylated RSKs in turn activate transcription factors, such as CREB, and are involved in cell cycle control, memory formation, and suppression of apoptotic cell death (Nebreda and Gavin, 1999). Moreover, inactivation mutations in the gene en-

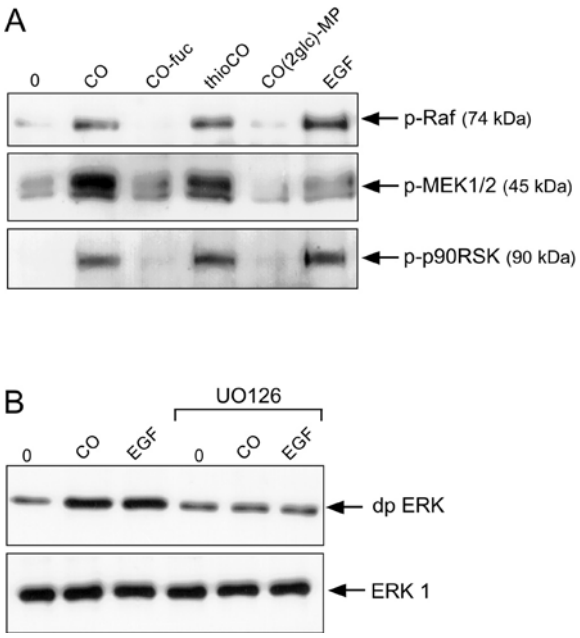

Figure 4. Signaling pathway involved in ERK activation by CO and CO derivatives. Serum-starved zebrafish cells (ZF13) were stimulated with 10^{-7} M of CO, CO derivatives, and EGF for 15 min. Lysates were subjected to western blotting with phosphospecific p-Raf, p-MEK1/2, and p-p90RSK antibodies (A). Inhibition of CO and EGF induced activation of ERK by MEK inhibitor. Serum-starved zebrafish cells (ZF13) were pretreated for 1 h with 10^{-5} M of a highly selective MEK1/2 inhibitor (UO126) prior to 15 min stimulation of ERK phosphorylation by 10^{-7} M CO and EGF (B). Phosphorylated and nonphosphorylated forms of ERKs were detected by dp ERK and ERK1 antibodies.

coding human RSK2 are associated with Coffin-Lowry syndrome, a disease that results in severe mental retardation as well as progressive skeletal deformation (Treisman, 1996). The recently discovered RSK4 is deleted in patients with X-linked mental retardation (XLMR) and may be a candidate XLMR gene (Yntema et al., 1999).

Our results strongly support the model (Fig.5), but final genetic identification of all components of CO signaling remain to be determined. Several lines of evidence in different cell types indicate that p90RSKs are activated by MAPKs *in vivo* via a Ras-dependent protein kinase cascade that is triggered by growth factors or tumor-promoting phorbol esters (Alessie et al., 1995). Moreover, other physiological substrates of p90RSK have been identified with different effects in development, cell cycle, suppression of apoptotic cell death, and progressive skeletal deformation (Treisman, 1996; Nebreda and Gavin, 1999). These findings promote future studies of p90RSK and its physiological

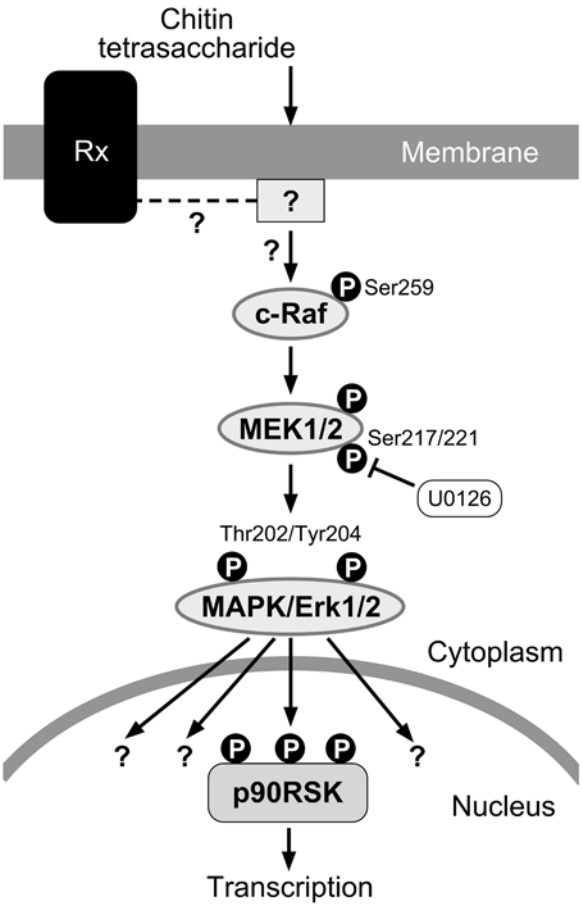

Figure 5. Model for specific activation of the ERK pathway by COs in embryonic zebrafish cells. Chitin tetrasaccharides probably activate a high-affinity receptor system (Rx) that transduces the signal to phosphorylation of Raf, MEK1/2, ERK1/2, and p90RSK. An arrow indicates an alternative path through the membrane.

substrates in zebrafish cells and embryos.

We recently cloned two zebrafish ERK genes in which the phosphorylation consensus is identical to the mammalian species and forms the expected epitope recognized by the dpERK antibody. With a morpholino knock-down approach we plan to find specific CO targets and study their function in zebrafish cells and embryo development. Recent studies in plants have identified leucine-rich repeat (LRR) receptors involved in recognizing carbohydrate microbial factors, such as the Nod factors (Asai et al., 2002; Stracke et al., 2002; Endre et al., 2002). LRR domains are also found in plants as part of disease

resistance cascades, where they function as receptors important for innate immunity. In animals, the relatives of the plant receptors, so called Toll-like receptors, are also involved in the recognition of microbes, meaning that a similar carbohydrate-recognition mechanism could underlie the plant and animal innate immune system (Beutler, 2002; Spaink, 2002). Recently we identified ~20 Toll-like receptors in the zebrafish database from Sanger Centre (unpublished data). Currently we study their expression during embryogenesis and in response to treatment of cell lines with different carbohydrates to elucidate their role in CO signaling cascade.

In future research we aim to identify CO receptors and link them to downstream pathways, such as the ERK pathway, to fully understand CO function in vertebrate development.

Materials and methods

Materials

Polyclonal phospho-p44/42MAP kinase antibody, phospho-ERK1/2 pathway sampler kit, phospho-p38 MAPK antibody, phospho-SAPK/JNK antibody, and MEK1/2 inhibitor (UO126) were purchased from Cell Signaling Technology (Leusden, The Netherlands). Control ERK antibody K-23 was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody, enhanced chemiluminescence-kit, and rainbow molecular weight markers were from Amersham Life Science (Roosendaal, The Netherlands). Murine EGF was obtained from Invitrogen (Breda, The Netherlands). Leibovitz L-15 medium (L-15) and fetal calf serum were purchased from Invitrogen.

Chitin tetrasaccharides were purchased from Seikagaku (Tokyo). Replacement of one or two central GlcNac residues of the chitin tetrasaccharide by Glc unit was received as described (Wang and Lee, 1995; Robina et al., 2002; Southwick et al., 2002). Fucosylation of COs by NodZ protein was performed as described previously (Bakkers et al., 1997).

The syntheses of thioCO

The syntheses of di-N-acetyl-4-thiochitobiose (TG_2), α-methyl di-*N*-acetyl-4-thiochitobioside (MTG_2), α-methyl tri-*N*-acetyl-4,4'-dithiochitotrioside (MTG_3), α-methyl tetra-*N*-acetyl-4,4',4''-trithiochitotetraoside (MTG_4), and β-methyl tri-*N*-acetyl-4,4'-dithiochitotrioside (β-MTG3) were reported in Wang and Lee (1995). The reducing thiochito-oligomers, tri-*N*-acetyl-4,4'-dithiochitotriose

(TG_3) and tetra-*N*-acetyl-4,4',4''-trithiochitotetraose (TG_4), were prepared as follows.

TG_3. The peracetylated 4,4'-dithiochitotriose (20 mg) (Wang and Lee, 1995) was de-*O*-acetylated with sodium methoxide in methanol (10 mM, 5 ml) at room temperature for 6 h. Water (1 ml) was added and the solution was neutralized with Dowex 50w-x8 resin (H^+ form). After removal of the resin by filtration, the filtrate was concentrated. The trisaccharide was dissolved in a small amount of water and applied to a Sephadex G-10 column (1.5 x 90 cm), which was pre-equilibrated and eluted with 50 mM acetic acid. Fractions containing the trisaccharide were pooled and lyophilized to give 15.5 mg TG_3 (yield: 81%). ^{1}H- nuclear magnetic resonance (NMR) of TG_3 (500 MHz, D_2O, 60°C): δ 5.248 (d, *J* = 3.4 Hz, 0.35 H, H-1α), 4.776 - 4.732 (two pairs of d, *J* = 10.2 Hz, 2 H, H-1',1''), 4.695 (d, *J* = 8.4 Hz, 0.65 H, H-1β), 4.057 - 3.470 (m, 16 H, sugar protons), 2.957 - 2.856 (two pairs of t, 2 H, *J* = 10.4 Hz, H-4,4'), 2.059 - 2.049 (singlets, 9 H, 3 N-acetyl).

TG_4. The α-methyl 4,4',4''-trithiochitotetraoside (4.5 mg) (Wang and Lee, 1995) was per-*O*-acetylated with acetic anhydride±pyridine (1:1, 1.5 ml) at room temperature for 5 h. Ice water (2 ml) was added, and the solution was evaporated to dryness. Trace of volatiles was removed by coevaporation of the residue with toluene (3 x 2 ml) to give 7.5 mg of the peracetylated α-methyl 4,4',4''-trithiochitotetraoside. The compound obtained was subjected to acetolysis with Ac_2O-AcOH-H_2SO_4 (8:2:0.1, 2 ml) to selectively remove the 1-*O*-methyl group. After acetolysis at room temperature for 12 h, the solution was cooled to 0°C, and a cold solution of aqueous sodium acetate (0.2 M, 3 ml) was added. The mixture was evaporated to dryness and the residue was partitioned between $CHCl_3$ (10 ml) and water (2 ml). The organic layer was separated and successively washed with 5% hydrochloric acid (1 ml), saturated sodium bicarbonate (1 ml), and water (2 x 1 ml), dried with anhydrous sodium sulfate, and filtered. The filtrate was evaporated to afford the peracetylated 4,4',4''-trithiochitotetraose as a white solid, which was subsequently de-*O*-acetylated with sodium methoxide in methanol (10^{-2} M, 5 ml). The product was purified by gel filtration with a Sephadex G-10 column the same way as described for the preparation of TG_3 to give 3.5 mg TG_4 (overall yield in three steps: 79%). ^{1}H-NMR of TG_4 (500 MHz, D_2O, 60°C): δ 5.246 (d, *J* = 3.4 Hz, 0.39 H, H-1α), 4.769-4.725 (3 pairs of d, *J* = 10.3 Hz, 3 H, H-1',1'',1'''), 4.693 (d, *J* = 8.0 Hz, 0.61 H, H-1β), 4.059- 3.467 (m, 21 H, sugar protons), 2.945-2.872 (three pairs of t, 3 H, *J* = 10.3 Hz, H-4,4',4''), 2.050-2.047 (singlets, 12 H, 4 *N*-acetyl).

Cell lines

Cell lines ZF13 and ZF29c-1 were obtained from the Hubrecht laboratory. These cells have been derived from dechorionated, disaggregated, 20 h-old zebrafish embryos. They have a fibroblast-like morphology (Peppelenbosch et al., 1995). For our experiments cells were grown at 25°C in 2 ml 67% L-15 medium supplemented with 10% fetal calf serum in 24-well plates (Greiner, Alphen aan de Rijn, The Netherlands) until the plates became 50% confluent. Before stimulation experiments, the medium was changed to serum-free medium (SFM) for 18-24 h to reduce the basal level of phosphorylation. This SFM was then replaced with 1 ml SFM plus indicated stimulus for various times. Stimulation was terminated by quick replacement of SFM for 600 μl, 65°C sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, 3% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). The cells were scraped off the dish, and the cell lysates were transferred to a new tube, boiled for 3 min, and separated on 10% polyacrylamide slab gels (10 μg protein per lane).

Western immunoblotting

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Den Bosch, The Netherlands) by western blotting. The membranes were blocked in 5% w/v nonfat dry milk in Tris-buffered saline-Tween 20 (TBST). The blots were incubated with a 1:1000 to 1:2000 dilution of the indicated antibody in TBST with 3% bovine serum albumin (Sigma, St. Louis, MO) for 1 h at room temperature or overnight at 4°C. Signal was detected using a 1:5000 to 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibodies and the enhanced chemiluminescence method (Amersham).

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