The Caenorhabditis elegans p21-activated Kinase (CePAK) Colocalizes with CeRac1 and CDC42Ce at Hypodermal Cell Boundaries during Embryo Elongation*

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The p21-activated kinase (PAK) is a downstream target of Rac and CDC42, members of the Ras-related Rho subfamily, that mediates signaling pathway leading to cytoskeletal reorganization. To investigate its function in Caenorhabditis elegans development, we have isolated the cDNA coding for the p21-activated kinase homologue (CePAK) from a C. elegans embryonic cDNA library. This 2.35-kilobase pair cDNA encodes a polypeptide of 572 amino acid residues, with the highly conserved N-terminal p21-binding and the C-terminal kinase domains. Similar to its mammalian and Drosophila counterparts, the CePAK protein expressed in E. coli exhibits binding activity toward GTP-bound CeRac1 and CDC42Ce. Polyclonal antibodies raised against the recombinant CePAK recognize a specific 70-kDa protein from embryonic extracts that displays CeRac1/CDC42Ce-binding and kinase activities. Immunofluorescence analysis indicates that CePAK is specifically expressed at the hypodermal cell boundaries during embryonic body elongation, which involves dramatic cytoskeletal reorganization. Interestingly, CeRac1 and CDC42Ce are found at the same location, which might point to their common involvement in hypodermal cell fusion, a crucial morphogenetic event for nematode development.

The embryos of multicellular organisms undergo profound cytoskeletal reorganization following fertilization (1). A striking example of such morphological changes is observed in the embryogenesis of the nematode Caenorhabditis elegans. In the second half of embryonic development, rapid cell proliferation largely ceases, whereas the length of the embryo increases by four times (2). Unlike plant cells (3) or insect oocytes (4) where elongation is achieved by limiting radial expansion of the symmetrical cell growth, C. elegans embryo maintains the same volume throughout embryonic development. Cytoskeletal reorganization is therefore essential for this developmental process. One noticeable feature during nematode embryo elongation is the shape changes of the hypodermal cells, which become elongated by fusing into each other (5). These cells contain contractile circumferentially oriented bundles of microfilaments (both actin fibers and microtubules), which are associated at the apical membranes, similar to the vertebrate adherens junctions (2). The body elongation is achieved by the shortening of these microfilaments as the hypodermal cells fuse. Interestingly, these microfilaments are only structurally organized during the elongation event.

Key molecules involved in cytoskeletal reorganization include the Rho subfamily of p21 Ras-related proteins. In mammalian cells, Rac induces the formation of lamellipodia (6), and Rho is responsible for the formation of stress fibers (7), whereas CDC42 promotes formation of filopodia (8, 9). In lower multicellular organisms such as Drosophila melanogaster, the Rho subfamily has been shown to be involved in various aspects of actin-dependent morphogenesis including dorsal closure (10) and epithelial cell shape changes (11). The activity of Rho p21s, like other Ras-related proteins, is modulated by different types of regulatory proteins (GTPase-activating proteins, GDP dissociation inhibitors, and guanine nucleotide exchange factors) resulting in the cycling of these p21s between an active GTP-bound form and an inactive GDP-bound form (12). A more promising downstream target is the Rac/CDC42-activated serine/threonine kinase PAK, recently characterized in our laboratory (13), and an increasing number of its isoforms has been identified (14–22). Its function as an effector is suggested not only by its ability to bind to the active GTP-bound forms of either Rac or CDC42 but more importantly by the fact that it becomes activated upon binding.

To investigate the role of PAK in morphogenesis during C. elegans embryogenesis, we have isolated in this study the nematode PAK homologue (CePAK), which displays conserved biochemical activities. CePAK whose mRNA is highly expressed in embryos, is specifically localized at the hypodermal cell boundaries throughout the embryonic body elongation. Interestingly, our study also shows that both CeRac1 (23) and CDC42Ce (24) colocalize with CePAK, therefore suggesting their common involvement in this crucial morphogenetic event.

EXPERIMENTAL PROCEDURES

Growth and Handling of C. elegans—The Bristol N2 strain of C. elegans was grown at 20 °C with Escherichia coli OP50 as a food source (23, 25). Worms of each developmental stage were obtained from cultures synchronized as described previously (23).

Cloning and Sequencing of CePAK cDNA—Degenerate oligos (LLWN91 and 92; see Fig. 1) were designed on the basis of sequence conservation between the Drosophila DPAK (22) and rat PAK (13). The resulting PCR fragment was used as probe to screen a C. elegans embryonic cDNA library (from Stratagene) under low stringency conditions as described previously (23). cDNA inserts of positive plaques were isolated by in vivo excision. The complete sequence of the cDNA

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¶ The abbreviations used are: PAK, p21-activated kinase; GST, glutathione S-transferase; PCR, polymerase chain reaction; DPAK, homologue of PAK in Drosophila.
clones was obtained on both strands using the Sequenase DNA sequencing kit (from U. S. Biological Corp.). Sequence analysis and comparison were performed using the DNAStar program package.

Extraction of Total RNA from C. elegans and Northern Blot Analysis—Total RNA from each developmental stage was isolated as described (23). Nylon filter containing these RNAs was subjected to hybridization under high stringency conditions (23) with a 5′ EcoRl BamHI fragment of CePAK cDNA as probe (Fig. 1).

Expression of CePAK in E. coli—The coding regions of CePAK was isolated by PCR using specific primers. The resulting fragment was restriced by SmaI prior to in-frame ligition into SmaI site of GST (glutathione S-transferase) expression vector pGEX-3 (Pharmacia Biotech Inc.). The fusion protein GST/CePAK was induced and purified as described (27).

Rho p21s Binding Assay—The nitrocellulose filters containing the purified GST fusion protein of the full-length CePAK were incubated overnight at 4 °C in renaturation solution (3% bovine serum albumin, 0.1% Triton X-100, 0.5 mM MgCl2, 5 mM dithiothreitol) (14). CeRac1, CDC42Ce, and CeRhoA (28) were labeled separately with either [γ-32P]GTP or [β-35S]GDP (both at 1200 Ci/mm, from DuPont NEN) as described (30). The binding of the radiolabeled Rho p21s to CePAK was then carried out as described (14).

Immunoprecipitation and in Situ Kinase Assay—Immunoprecipitation experiment was carried out as described (27) with 800 μg of the cytosolic fraction of mixed-stage population (29), incubated with either 20 μg of the affinity-purified CePAK antibody (see below) or preimmune serum. The supernatant containing the immunoprecipitation complex was subjected to the in situ kinase assay by incubating for 30 min at room temperature in 1 ml of 40 mM Hepes, pH 7.4, 10 mM MgCl2, 2 mM MnCl2, 50 μg/ml [γ-32P]ATP (3000 Ci/mmol, from DuPont NEN) containing either cold γGTP-CeRac1 or cold γGTP-CDC42Ce labeled as described for the Rho p21s binding assay. The filters were washed as described (21). p21 binding assay was separately carried out on these filters using either [γ-32P]GTP-CeRac1 or [γ-32P]GTP-CDC42Ce as described above. Filters were then autoradiographed overnight at −70 °C.

Particulate and Cytosolic Fractionation of C. elegans—Embryos of C. elegans were collected by the alkaline hypochlorite method as described (25). They were then resuspended in phosphate-buffered saline plus 4 μg/ml leupeptin, 4 μg/ml aprotinin, and 4 μg/ml pepstatin. The suspension was sonicated, and the cytosolic and particulate fractions were prepared by high speed centrifugation as described (29).

Polyclonal Antibody against CePAK, CeRac1, and CDC42Ce and Western Immunoblot Analysis—Immunizations of rabbit were carried out as described (29) by injecting into rabbits the GST fusion proteins of a partial CePAK coding region, issued from cloning the PCR fragment with LLWN149 and 124 as primers (Fig. 1) into pGEX-3 plasmid, CeRac1 and CDC42Ce previously expressed and purified in E. coli (23, 24). The antibodies were subsequently affinity-purified using the ImmunoPure Ag/Ab Immobilization Kit (Pierce) as described (29). Western blot analysis using both cytosolic and particulate embryonic extracts was carried out as described (29) with the affinity-purified antibodies against CePAK, CeRac1, and CDC42Ce, respectively. Antigen-antibody complexes were detected using the enhanced chemiluminescence kit (Amersham Corp.). To determine the specificity of the CeRac1 and CDC42Ce antibodies, nitrocellulose filters containing both 2 μg of CeRac1/GST and 2 μg of CDC42Ce/GST proteins were prepared as above and incubated with CeRac1 and CDC42Ce antibodies separately.

Indirect Immunofluorescence Analysis on C. elegans Embryos—Embryos of C. elegans were collected and treated as described (25). They were resuspended in phosphate-buffered saline plus 0.05% Tween 20 and 2% dried milk powder (solution A) and incubated overnight at 4 °C with 0.5 μg of each of the following antibodies: CePAK, CeRac1, CDC42Ce, and MH27 (recognizing exclusively the hypodermal cell boundaries). These embryos were then incubated for 2 h at room temperature with Indocarbocyanine (C3)-coupled anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.), whereas those treated with CePAK antibody were incubated with Rhodamine-coupled anti-mouse antibody (kindly provided by Dr. Nick Harden in our laboratory). The embryos were finally resuspended in the mounting solution (29) and examined through fluorescence microscopy using a Zeiss Rhodamine filter 487715. Kodak Ektachrome color films were used for photography. Negative controls include the secondary antibody alone, preimun serum, and the antibodies against CePAK, CeRac1, and CDC42Ce that were blocked with excess of the respective GST fusion proteins.

RESULTS AND DISCUSSION

Sequences within p21-binding and Kinase Domains Are Highly Conserved in CePAK—The p21 Rac/CDC42-activated kinase (PAK) has been shown to be a direct target of these members of the Rho subfamily because it binds exclusively to their active GTP-bound form, and the kinase activity is greatly enhanced upon binding to these Rho p21s (13). To investigate its involvement in C. elegans development and establish a biological link to CeRac1 and CDC42Ce previously isolated in our laboratory (23, 24), we set out to isolate the nematode PAK homologue. As a first step, we designed degenerate oligos LLWN91 and LLWN92 based on the highly conserved kinase domains between the prototype rat a-PAK (13) and its Drosophila homologue (22) (Fig. 1). The sequence of the 150-base pair fragment issued from PCR using a C. elegans mixed-stage cDNA library indicated its identity as part of the putative nematode PAK cDNA. This fragment was then used as probe to screen a C. elegans embryonic cDNA library (from Stratagene). Four clones were isolated from 2 × 107 plaques, and inserts were cloned into Bluescript SK plasmid via in vitro excision. Sequence analysis indicated that the largest clone of 2348 base pairs contained an open reading region coding for 572 amino acid residues with a predicted molecular size of 64 kDa (Fig. 1), whereas the three others were partial clones of identical sequence (data not shown).

At the amino acid sequence level, the deduced CePAK polypeptide displayed an overall 52.8 and 53.5% sequence similarity compared with DPAK and rat a-PAK, respectively. However, the similarity level was significantly higher within the two conserved domains; 75 and 72% sequence identity were found in the N-terminal p21-binding domain (spanning 57 amino acid residues; Fig. 2) between CePAK and DPAK and between CePAK and rat a-PAK, respectively, whereas the C-terminal domain (spanning 275 amino acid residues; Fig. 2) of CePAK displayed 80 and 76% identity compared with that of DPAK and rat a-PAK, respectively. This high degree of sequence conservation therefore suggests an important role for PAK in animal development.

In collaboration with Dr. A. Coulson (Medical Research Council, UK), the CePAK cDNA was mapped to chromosome X in close proximity to kin-2 coding for the regulatory subunit of a cAMP-dependent protein kinase (32). The mapping result was further confirmed by the C. elegans genome sequencing project in which the CePAK gene was identified at the same location (Genbank accession number U29612).
GST fusion protein with the partial coding region of the CePAK cDNA (using LLWN149 and 127 as primers in the polymerase chain reaction; Fig. 1). A single band of 70 kDa, which was present in both the cytosolic and particular fractions of the embryonic extracts, was recognized by the affinity-purified antibody (Fig. 4); this was close to the size of CePAK expressed in E. coli and may therefore represent the endogenous CePAK.

Immunoprecipitation was then carried out by incubating this antibody with the cytosolic extract of a mixed stage population of C. elegans, and the complex was separated on SDS-polyacrylamide gel prior to the in situ p21 binding and kinase assay. Results indicated that a 70-kDa protein from the complex between CePAK antibody and the C. elegans extracts, which might be the endogenous CePAK, was able to bind the GTP-bound CDC42Ce (Fig. 5, lane 2) and the GTP-bound CeRac1 but not the GTP-bound CeRhoA (data not shown). More importantly, this 70-kDa protein also exhibited kinase activity in the presence of the GTP-CDC42Ce (Fig. 5, lane 1) and GTP-CeRac1 (data not shown). As a control, no activity in either the p21 binding or the kinase assays was detected from the immunoprecipitation complex between the preimmune serum and the C. elegans extract (Fig. 5, lanes 3 and 4). Our results therefore indicated that CePAK displayed biochemical activities that were conserved in its homologues from other organisms.

CePAK mRNA Is Highly Expressed in Embryogenesis—We have previously shown by Northern blot analysis that all three members of the C. elegans Rho subfamily were highly expressed during embryonic development (23, 24, 29). To determine the developmental expression pattern of CePAK, Northern blot analysis on total RNA extracted from six developmental stages was carried out using a EcoRI/BamHI fragment (containing the 520-basepair 5' region of CePAK; Fig. 1) as probe. Results under high stringent hybridization conditions indicated the presence of a 2.4-kilobase pair mRNA, corresponding to the size of CePAK cDNA, throughout development (Fig. 6A). However, its relative abundance measured by normalizing the signal to that of the nematode actin gene at the corresponding stage (Fig. 6B) indicated that CePAK was highly expressed at embryonic stage (Fig. 6C). Despite a similar expression pattern to the three Rho p21s, the decrease of the CePAK mRNA level from L1 stage onward was more dramatic and probably reflects an even more important role of CePAK in...
Embryogenesis.

CePAK Is Specifically Localized at Hypodermal Cell Boundaries during Embryo Elongation—Embryogenesis in *C. elegans*, which takes 14 h from fertilization to hatching, can be divided into two distinct phases (2). The first phase of this is marked by rapid cell proliferation with two-thirds of the total somatic cells generated. In contrast, morphogenesis is the most prominent event during the second phase of embryogenesis, whereby virtually no cell division is detected but the embryo body has elongated 4-fold. Such an elongation event involves dramatic cytoskeletal reorganization because the *C. elegans* embryo remains at a constant volume during the entire process.

To investigate further the function of CePAK in embryogenesis as suggested by its high level of mRNA, its tissue distribution was analyzed by indirect immunofluorescence using the affinity-purified anti-CePAK antibody. Whereas no signal was detected in early embryogenesis where cell proliferation takes place, CePAK was specifically localized at all hypodermal cell boundaries throughout the second phase of embryogenesis (Fig. 7A–E). The identity of these cells as hypodermal cells was supported by the identical staining pattern of the MH 27 monoclonal antibody, which exclusively recognizes hypodermal cell boundaries (kindly provided by Dr. Cousu-Hresko, University of Washington) (Fig. 7F). Hypodermal cells have been shown to be responsible for embryonic body elongation by squeezing the circumferentially oriented microfilament bundles attached to adherens junctions at their apical membranes (2, 5). During the elongation event, boundaries of the hypodermal cells disappear progressively as the distance between the longitudinal margins of each cell increase, whereas between the circumferential margins decrease. The CePAK staining pattern coincided with these cell shape changes, as illustrated from an embryo at the beginning of elongation (Fig. 7A) when all hypodermal cell boundaries were still present (arrowhead) to an elongated embryo (Fig. 7E) where only two continuous longitudinal boundaries were observed (arrowheads). Despite their identical staining pattern, it is unlikely that the CePAK antibody cross-reacts with MH27 antibody because the latter recognizes a much larger 150-kDa protein (33, 34). As a negative control, no signal was detected in similar immunofluorescence analysis using the CePAK antibody, which was blocked by the GST/CePAK fusion protein (data not shown).

Our results therefore suggest an involvement of CePAK in the dynamic cell shape changes during embryonic body elongation, with a possible function in the reorganization of adherens junctions. In support of this involvement, the homologue of PAK in *Drosophila* (DPAK) has been implicated in the actin assembly at adherens junctions (22). DPAK is highly expressed in the leading edge of epidermal cells whose shape changes are responsible for dorsal closure, a morphogenetic event in which Rh21 is also implicated (10, 22). Interestingly, a transient loss of both DPAK and the components of adherens junctions (i.e. actin and myosin) was observed during dorsal closure (22). This may be correlated to hypodermal cell fusion in *C. elegans*, in which adherens junctions at cell boundaries (where CePAK was localized) disappear progressively, and therefore points to a conserved mechanism in cell shape changes mediated by PAK in these organisms.

**CeRac1 and CDC42Ce May Participate with CePAK in Embryogenesis.**

**Fig. 2.** Alignment of the amino acid sequence of CePAK with rat (PAK) and *Drosophila* (DPAK) homologues. Residues identical to CePAK are shaded, and insertions are denoted by a dash. Positions within each protein are indicated by numbers on the left side. The N-terminal box represents the p21-binding domain, and the C-terminal kinase domain is boxed.
were blocked with their respective GST fusion proteins (data not shown). No signal was detected using these antibodies when they were found to localize with CePAK at the cell boundaries (Fig. 7A). The specificity of these two antibodies was demonstrated by control Western blot analysis with either recombinant Ce-Rac1 or Ce-CDC42 proteins, which showed no cross-reactivity of the antibodies (data not shown). Indirect immunofluorescence analysis using these two antibodies indicated that these two Rho proteins were ubiquitously expressed throughout embryogenesis (data not shown), as would be expected from their known involvement in morphogenesis and development (6–11). However, at the stage when hypodermal cell fusion was occurring both CeRac1 (Fig. 7C) and CeCDC42 (Fig. 7H) were found to localize with CePAK at the cell boundaries. No signal was detected using these antibodies when they were blocked with their respective GST fusion proteins (data not shown). This co-localization suggests that these two p21s may participate with PAK in embryonic body elongation. In contrast, similar analysis using the CeRhoA antibody raised previously (29) showed that CeRhoA was not detected in cell boundaries throughout embryogenesis (data not shown). Taken together, our localization results disclose a possible functional link between CeRac1 and CeCDC42 and their downstream target (CePAK) in a common morphogenetic process, similar to the involvement of both DRac and DPAK in dorsal closure in Drosophila (22). The localization of these two p21s at the hypodermal cell boundaries (possibly at adherens junctions) is also consistent with the findings in Drosophila implicating both DRac and DCDC42 in epithelial cell shape changes, with DRac involved in the recruitment of actin at adherens junctions and DCDC42 in polarized cell shape changes (11).

In conclusion, we have shown in this study that the sequence of C. elegans p21

Rac1/CDC42-activated kinase (CePAK) is highly conserved within the p21-binding and kinase domains. This sequence conservation is also reflected in its conserved biochemical activities. An involvement of CePAK in C. elegans embryogenesis is indicated by its high mRNA level in embryos and more importantly by its localization to the hypodermal cell boundaries during embryonic body elongation. The colocalization of the CeRac1 and CeCDC42 with CePAK is consistent with our in vitro binding results as well as with these GTPases...
being activators of PAK (13) and may indicate their common involvement in morphogenetic events during C. elegans development.

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Fig. 7. Analysis of in situ expression of CePAK, CeRac1, and CDC42Ce by immunofluorescence. Embryos were prepared for immunofluorescence analysis as described. Panel A shows the dorsal view of an embryo at the beginning of body elongation; all the hypodermal cell boundaries are stained by the CePAK antibody. The arrowhead points to one circumferential boundary. Panels B–E represent the staining pattern of embryos undergoing hypodermal fusion and body elongation. Panel B is the lateral view of an embryo where the staining can be seen at both circumferential (indicated by small arrowhead) and longitudinal (indicated by big arrowhead) cell boundaries. Panel C is the lateral view of an embryo at a later stage of the elongation event (compared with that in panel B), where the staining of the circumferential boundary disappears but that of the longitudinal boundary remains clearly visible (arrowhead). Panel D is the lateral view of an embryo at the end of the elongation event. Only the longitudinal boundaries are stained (indicated by two arrowheads) as all circumferential ones have fused into each other. Panel E is the dorsal view of an embryo at the about same stage as that in panel D. The two longitudinal cell boundaries can be seen (indicated by arrowheads). Panel F is the lateral view of an embryo stained by MH 27 monoclonal antibody, which exclusively recognizes the hypodermal cell boundaries. Panel G is the dorsal view of an embryo, which is at the late stage of the body elongation, stained with the affinity-purified anti-CeRac1 antibody (see also panel I). The two longitudinal boundaries (indicated by arrowheads) are visible, similar to the staining pattern of CePAK in panel E. Panel H is the lateral view of an embryo during the elongation event stained with the affinity-purified anti-CDC42Ce antibody (see panel I). Staining of both the circumferential (indicated by small arrowhead) and the longitudinal boundaries (indicated by big arrowhead) were observed. Panel I shows the Western blot analysis with the affinity-purified anti-CeRac1 and anti-CDC42Ce antibodies raised in this study. Conditions were identical as for the analysis of the CePAK antibody (see Fig. 4), except that the cytosolic (lane S) and particulate (lane P) fractions here are prepared from mixed stage populations of C. elegans as described (29). The sizes of the Rainbow protein markers (Amersham Corp.) are indicated on the left side of the filter.
C. elegans PAK in Hypodermal Cell Boundaries


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