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Proteolytic Cleavage of a Spectrin-Related Protein by Calcium-Dependent Protease in *Neurospora crassa*

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Abstract. To investigate the functional significance of a cytoskeletal spectrin-like protein, we studied its localization pattern in *Neurospora crassa* and sought the answer to whether it is a substrate for another apically localized protein, the calcium-dependent protease (CDP II). Immunoblots of crude extracts from exponentially growing mycelia, separated by one- and two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis using antichicken α/β-spectrin antibodies, revealed a single band of approximately relative mass (Mr) 100 kDa with an isoeletric point (pI) in the range of 6.5 to 7.0. Despite rigorous efforts, we could not confirm the presence of an Mr 240- to 220-kDa spectrin-like protein in *N. crassa*. The immunofluorescence- and immunogold-labeling Mr 100-kDa protein showed its predominance along the plasma membrane of the conidia during the swelling phase of germination. In contrast, in the germ tubes and the growing hyphae, the localization was polarized and concentrated mainly in the apical region. The *in vitro* proteolysis experiments showed that indeed this protein is a preferred substrate of CDP II which is, as mentioned previously, also localized in the apical regions of the hyphae. These results indicate a putative functional relationship between these two proteins (spectrin-like protein and CDP II) in the dynamics of tip growth.

The filamentous fungi represent a large group of organisms that grow and colonize substrates by branching and apical elongation. Cytoskeletal elements are considered to be involved in this process and have been the subject of intensive investigation. Ultrastructural and immunologic studies of the apical zone, for example, have shown that this region is rich in cytoskeletal elements, such as actin [1–4], tubulins [5–11], integrins, and spectrins [12–15]. These proteins are considered to provide stability to the growing hyphal tubes by the linkage among themselves and to the plasma membrane. For the hyphae to grow, this linkage must be reorganized.

Another important factor implicated in regulation of polarized growth and branch formation is calcium signaling [16–19]. We have reported the presence of two calcium-dependent proteases (CDPs I and II) in the apical region of *Allomyces*, an aquatic fungus, and shown that their presence or absence is related to growth [4, 20–24]. An immunoanalogue of CDP II was also discovered and shown to be exclusively localized in the apical regions of the hyphae of an ascomycete fungus, *Neurospora crassa*, and a phytopathogenic basidiomycete, *Uromyces appendiculatus* [25]. Therefore, this colocalization of cytoskeletal proteins and CDP might have some functional significance. Indeed, we have shown selective proteolysis of α-tubulin by CDP II in the apical region of the growing hyphae and cell-free extracts [24].

We sought to determine whether other apically localized cytoskeletal proteins are also targets of this protease. Because a spectrin-like protein has been shown to be localized in the apex of the growing hyphae of *N. crassa* using antichicken α/β spectrin antibodies [14], we re-examined the spatial localization of this protein and CDP II during early development of *N. crassa* and studied the proteolysis of the former *in situ* as well as in cell-free extracts by this protease.

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Materials and Methods

Organism and culture conditions. A wild-type strain of *N. crassa* (FGSC 262, strain St. Lawrence STA4) was used in this study. Production of conidial inocula and culture growth conditions were as described previously [26].

Protein extraction and analysis. Crude extract was prepared according to Abe and Davies [27] with slight modifications. Briefly, the buffer contained 5 mM HEPES-KOH at pH 7.5, 250 mM sucrose, 15 mM $Mg(OAc)_2$, 2 mM EGTA, 2 mM dithiothreitol, 25 mM $K_2O_5S_2$, 10% glycerol, 0.5% polyvinylpyrrolidone, and protease inhibitors (1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each for leupeptin, chymostatin, and trasylol). Protein concentration was determined according to Bradford [28]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional analysis were done according to Laemmli [29] and O'Farrell [30], respectively.

Immunoblotting. Proteins from unstained monodimensional and bidimensional gels were electrophoretically transferred to nitrocellulose membranes (BA85, pore size 0.45 μm), using the transfer buffer described by Burnette [31], and immunoblotted as described earlier [24]. Antichicken α/β spectrin (Sigma S1390) and antirabbit IgG antibodies coupled to horseradish peroxidase were used at dilutions of 1:800 and 1:2000 in Tris-buffered saline, respectively.

Immunofluorescence microscopy. Conidia were suspended in Vogel's growth medium, and a sample was taken immediately, which represented the start point (0 hours). Further samples were taken at 6, 12, and 18 hours of culture growth and fixed instantaneously by the addition of paraformaldehyde in the culture medium to a final concentration of 3%.

Primary antichicken spectrin antibodies and secondary goat antirabbit fluorescein isothiocyanate (green, Sigma F9887) or crystalline tetramethylrhodamine isothiocyanate (red, Sigma T5268) antibodies were used at dilutions of 1:50 and 1:100, respectively, in phosphate buffer [24]. These two secondary antibodies were used to differentiate the distribution of spectrin-like protein and CDP II in the colocalization experiments. The immunolabeled cells were examined with a microscope (Zeiss axioplan) equipped for epi-illumination with fluotar optics and selective filter combinations. Spectrin-like protein was revealed using Sigma antichicken α/β spectrin and anti-CDP II using laboratory stock of anti-CDP II prepared from *Allomyces arbuscula.* Colocalization was done using respective primary antibodies. The images were taken with a Hamamatsu color, chilled 3 CCD camera, developed by Raster Optics video captor and treated by the program Adobe PhotoShop 7.

Electron microscopy and immunogold labeling. Spores (0 hours) and 6-hour germinated conidia were used for immunogold labeling of the spectrin-like protein using antichicken spectrin antibodies diluted to 1:50 in phosphate buffer and secondary goat antirabbit antibodies conjugated to 20 nm gold particles and diluted to 1:30 in the same buffer. The experimental procedure used has been described in detail elsewhere [24]. Sections were examined at 60 kV using a Philips M400 transmission electron microscope.

Proteolysis of cell-free extract by CDP II. The cell-free extract, obtained as described in the section on protein extraction and analysis, was digested with CDP II (laboratory stock purified from *A. arbuscula* as described in Ojha and Wallace [20], specific activity 21 μM paranitroaniline released $\mu g^{-1} min^{-1}$) in a reaction mixture containing 20 mM Tris-HCl at pH 7.4, 4 mM EGTA, 3 mM $MgCl_2$, and 1% β-mercaptoethanol. 6 mM $CaCl_2$ was added to obtain 5 mM free Ca^{2+} to initiate the proteolytic activity. The reaction was performed in a total volume of 75 μl at enzyme-to-protein ratios of 1:100, 1:50, and 1:10. After incubation at 37°C for 30 minutes, the reaction was stopped by the addition of 25 μl 4 × loading buffer [29]. The proteins were denatured in boiling water for 5 minutes and separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunorevealed with spectrin polyclonal antibodies. Reaction mixtures either without calcium or with leupeptin (10 μM), an inhibitor of CDP II activity, were used as controls.

In situ proteolysis by CDP II. Hyphae from 12-hour cultures were prepared as described previously for immunofluorescence. Before blocking, the cells were incubated for 1 hour at 37°C with CDP II in the enzyme reaction buffer described previously, and washed with phosphate buffer five times for 5 minutes each, and then immunorevealed as described earlier [24].

Results

Proteins from the cell-free extract, separated in nondenaturing PAGE and immunoblotted with chicken antispectrin polyclonal antibodies, showed two immunoreacting bands (Fig. 1A). The lower band (arrow) from nondenaturing gel was cut and migrated in SDS-PAGE; a single strong band of Mr 100 kDa appeared (data not shown). In direct SDS-PAGE of the cell-free extract, a single heavily stained band of Mr 100 kDa was also observed. The intensity of this band did not change significantly in preparations obtained from cells either during germination or exponential growth (Fig. 1B). Human erythrocyte spectrin, used as a positive control, revealed two bands of Mr 240 and 220 kDa each (Fig. 1C).

The specificity of immunoreaction was demonstrated using human erythrocyte spectrin in a competition assay. The results showed a progressive diminution of the immunoreacting band with increasing concentration of the competing human spectrin (Fig. 1D). In two-dimensional gels, the predominant immunoreacting band was Mr 100 kDa, with a pI in the range of 6.5 to 7.0, in addition to a faint 80-kDa band with similar pI, representing probably a degradation product of the major immunoreacting protein (Fig. 1E).

Immunofluorescence of the protein revealed with polyclonal antispectrin antibodies was intense all around the peripheral regions of the isodiametric germinating conidia, representing the swelling phase of germination (earliest visible phase) (Fig. 2A). With the appearance of germ tube outgrowth representing the polarized growth phase, much of the fluorescence was concentrated in the plasma membrane of the germ tubes (Fig. 2B). In the cells from the 12- and 18-hour cultures, representing filamentous hyphal growth and corresponding to exponential growth phase, the fluorescence was also polarized and concentrated as a cap in the hyphal tips and branch initials (Figs. 2C through 2E).

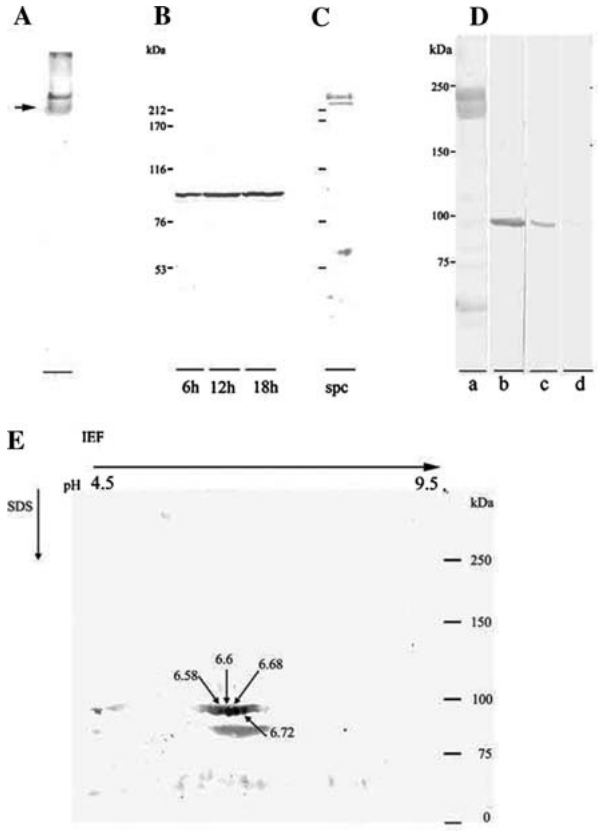

Fig. 1. Immunoblot of a *N. crassa* spectrin-like protein revealed with anti–α/β-spectrin antibodies. (A) Native PAGE of crude extract proteins; arrow indicates the band that gives the 100-kDa peptide in the SDS-PAGE gel. (B) SDS-PAGE of crude extract proteins from 6, 12, and 18 hours of culture development. (C) Human erythrocyte spectrin as control (1 μg). (D) Specificity assay of spectrin polyclonal antibodies in *N. crassa* with antibodies preadsorbed to human erythrocyte spectrin: (a) human erythrocyte spectrin revealed with unadsorbed antibodies; (b) crude extract with unadsorbed antibodies; (c and d) antibodies preincubated with human erythrocyte spectrin at ratios of 1:0.8 and 1:2, respectively. Preincubation of antibodies was performed for 30 minutes at room temperature before using them in immunoblot reactions. (E) Two-dimensional analysis of a *N. crassa* spectrin-like protein revealed with anti α/β-spectrin antibodies. The pI range of the protein is 6.5 to 7.0.

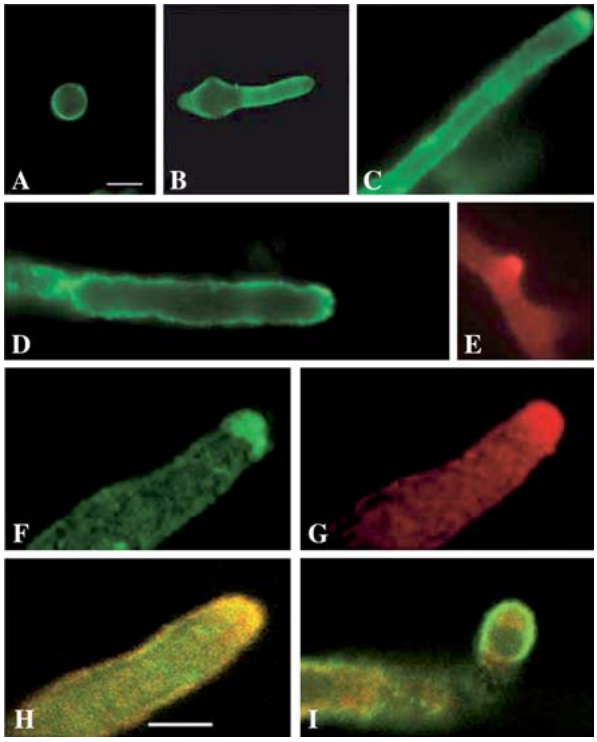

Fig. 2. (A through E) Immunolocalization of a *N. crassa* spectrin-like protein revealed with polyclonal antichicken α/β-spectrin in cells of different stages of growth. (A) Conidia. (B) Germinating conidia with germ tube. (C and D) Growing hyphae. (E) Tip of branch initial. Bar = 10 μm. (F through I) Colocalization of CDP II and spectrin-like protein in *N. crassa* hyphae. (F) Localization of spectrin-like protein. (G) Localization of CDP II. (H) Merge of two proteins in the tip of the germ tube. (I) Merge of two proteins in the branch initials. Bar = 10 μm.

In the colocalization experiments shown in Figs. 2F through 2I, both spectrin-like protein (green) and CDP II (red) in 6-hour germinating conidia and branch initials of the hyphae, were abundantly present in the same region of the cell, i.e., the growing tip. There was, however, clearly one difference: the predominance of spectrin-like protein along the plasma membrane and a more diffuse distribution of CDP II in the cytoplasm as evident in the merge of the localization of the two proteins (Figs. 2H through 2I).

Immunogold labeling the protein in ultrathin sections of conidia in the isodiametric and polarized phases of germination showed uniform distribution in the cortical region and along the plasma membrane (Fig. 3). Control experiments with antichicken antibody preadsorbed with human spectrin, or the use of only secondary antibodies, showed an absence of labeled grains indicating the specificity of the primary antibodies (data not shown).

Proteolysis of 100-kDa protein by CDP II in the presence of Ca^{2+} was evident by the diminution of the relative intensity of the band at low enzyme-to-protein ratios (1:100 and 1:50) and complete absence at the ratio of 1:10 (Fig. 4A, c through e). Under similar experimental conditions, but in the absence of Ca^{2+} or in the presence of leupeptin, this protein remained unaffected (Fig. 4A, f through g). Incubation of the extract without CDP II did not show any appreciable proteolysis of the immunoreacting protein, indicating there was no other endogenous protease targeting this protein under the test condition used. An examination of the relative intensity of Ponceau-stained bands after transfer from SDS-PAGE

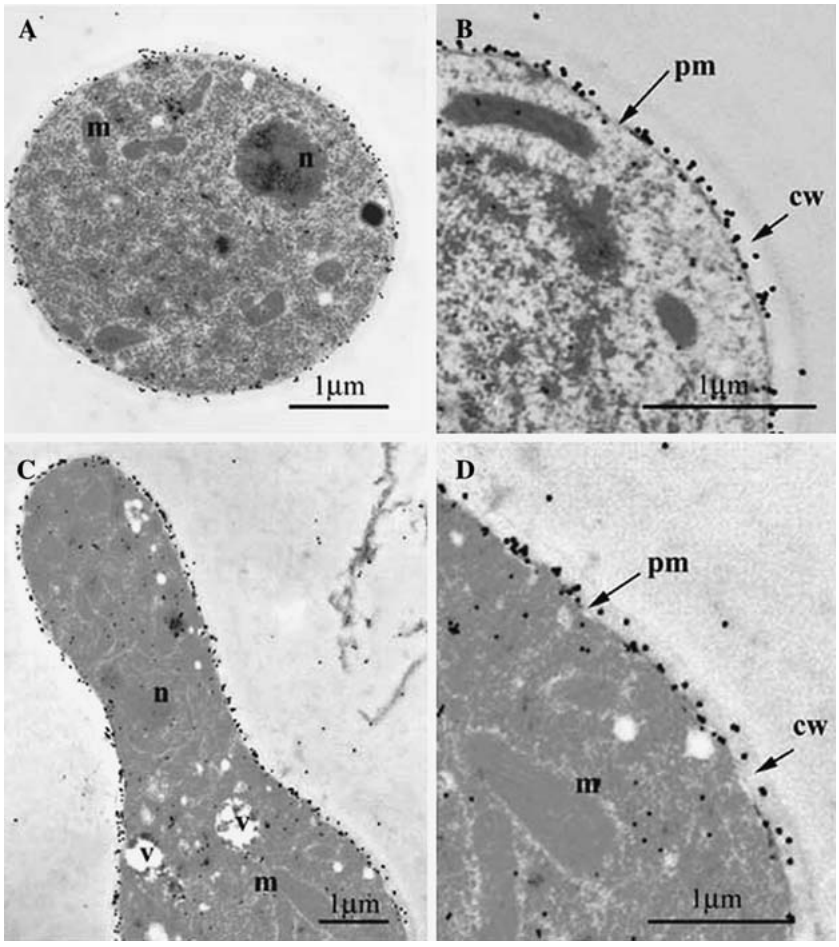

Fig. 3. Immunogold labeling of a *N. crassa* spectrin-like protein revealed with anti–α/β-spectrin antibodies during development. (A) Ultrathin section of conidia showing isodiametric distribution along the plasma membrane. (B) Distribution along the plasma membrane shown at a higher magnification of a portion (A). (C) Labeling along the plasma membrane in a section of conidia after 6 hours of germination. (D) Higher magnification of a portion of (C). m = mitochondria; n = nuclei; pm = plasma membrane; v = vacuole; cw = cell wall.

to nitrocellulose membrane did not show any massive general proteolysis.

Fixed and permeabilized cells incubated with CDP II in the enzyme reaction mixture and processed for immunofluorescence using antispectrin antibodies showed partial digestion of this protein where it appeared as faint fluorescent dots (Fig. 4C) instead of continuous fluorescence along the plasma membrane and capped localization at the tip observed in untreated cells (Fig. 4B). This pattern was similar to the one noted in our previous work with tubulins, in which β-tubulin, which is not affected by CDP II, had no modification in its hyphal distribution, whereas α-tubulin appeared as faintly dispersed fluorescent dots in the cytoplasm.

Discussion

A search in the genomic database of *N. crassa* for the spectrin superfamily proteins yielded two genes, the NCU06429.2 and NCU03992.2. The gene NCU06429.2 codes for a hypothetical protein corresponding to α–actinin, i.e., theoretical Mr 110 kDa. This protein has a C-terminal prodomain, two calponin homology domains, a rod domain composed of two spectrin repeats, and a N-terminal EF band domain. The gene NCU03992.2 codes for a protein (i.e., theoretical Mr 72 kDa) corresponding to fimbrin and comprised of four calponin homology domains and no spectrin repeat units. Western blot analysis of the cell-free extracts of *N.* crassa—using the same primary antibody that was previously used to recognize spectrin epitopes in plants [32–36], i.e., the oomycetes *Saprolegnia ferax* [12] and green algae [37]—yielded evidence for the occurrence of only one related protein of Mr 100 kDa. This protein could correspond to the gene product NCU06429.2. Contrary to published results [14] using the same antibodies and the same organism (*N. crassa)*, we did not find the presence of an Mr 240- to 220-kDa protein.

The spatial localization of the Mr 100-kDa protein by immunofluorescence showed that it is localized in the apical zone of the fungal hyphae (especially along the plasma membrane) confirming the published results [14]. The plasma membrane localization of the spectrin-like protein was more evident in the images obtained

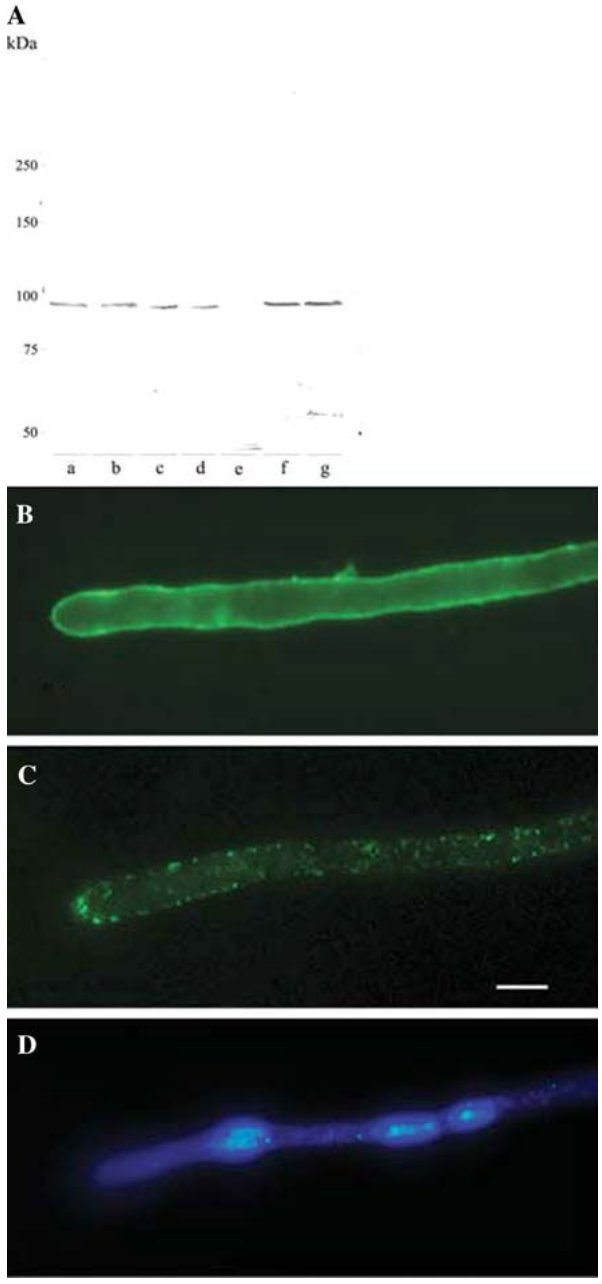

Fig. 4. (A) Proteolysis of a spectrin-like protein in the crude extract of *N. crassa* (50 μg/slot) with CDP II revealed by immunoblotting. The reactions were carried out for 30 minutes at 37°C, and the membrane was revealed with antibodies against α/β-spectrin. Lane a = Extract incubated without enzyme and calcium. Lane b = Extract incubated without enzyme but with calcium. Lanes c through e = Extract incubated with enzyme to protein ratios 1:100 (c), 1:50 (d), and 1:10 (e), all containing 5 mM free calcium. The controls were reaction mixture containing enzyme to protein 1:10 minus calcium (f) and enzyme-to = protein at ratio 1:10 plus calcium and leupeptin (g). (B through D) In situ digestion of a spectrin-like protein with CDP II. (B) Localization of spectrin-like protein along the plasma membrane. (C) After digestion with CDP II protease. (D) 4;6-Diamidino-2-phenylindole·2HCl (DAPI)-staining nuclei. Bar = 10 μm.

from the immunogold-labeling experiments. This localization may confer stability to plasma membrane by anchoring it to other cytoskeletal proteins. It is to be expected that proteolysis is necessary to weaken the attachment of cytoskeletal proteins to plasma membrane or other underlying hyphal tube structures and permit the expansion of the growing hyphal apex. Proteolysis of this plasma membrane–associated protein and possible weakening of the membrane skeleton may also facilitate the access of intracellular vesicles to the plasma membrane and allow its expansion. This is of importance because the tip of the hyphae has been shown to be a site of membrane insertion by exocytosis processes [38, 39]. The question arises, what is the identity of the protease responsible for this proteolysis? The apical localization of CDP II, as shown in Fig. 2H, essentially confirmed our previous finding reported earlier [25]. The colocalization of the spectrin-like protein and CDP II in the *N. crassa* hyphal apices (Fig. 2I) suggests that this spectrin-like protein at some point in development is proteolyzed by CDP II and regulated in its function.

Considering the in vitro and in situ proteolysis of this spectrin-like protein and its colocalization with CDP II, we suggest that it is a likely candidate for one of the specific substrates in vivo.

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