Candida albicans β-Glucan Exposure Is Controlled by the Fungal CEK1-Mediated Mitogen-Activated Protein Kinase Pathway That Modulates Immune Responses Triggered through Dectin-1

Marta Galán-Díez, David M. Arana, Diego Serrano-Gómez, Leonor Kremer, José M. Casasnovas, Mara Ortega, Álvaro Cuesta-Domínguez, Angel L. Corbí, Jesús Pla and Elena Fernández-Ruiz


Updated information and services can be found at:
http://iai.asm.org/content/78/4/1426

These include:

SUPPLEMENTAL MATERIAL
Supplemental material

REFERENCES
This article cites 59 articles, 23 of which can be accessed free at: http://iai.asm.org/content/78/4/1426#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Candida albicans β-Glucan Exposure Is Controlled by the Fungal CEK1-Mediated Mitogen-Activated Protein Kinase Pathway That Modulates Immune Responses Triggered through Dectin-1

Marta Galán-Díez,1 David M. Arana,2 Diego Serrano-Gómez,1 Leonor Kremer,3 José M. Casasnovas,4 Mara Ortega,1 Álvaro Cuesta-Domínguez,1 Angel L. Corbi,5 Jesús Pla,2 and Elena Fernández-Ruiz1*

Unidad de Biología Molecular, Hospital Universitario de la Princesa,1 Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid,2 Departamento de Inmunología y Oncología,3 and Departamento de Estructura de Macromoléculas,4 Centro Nacional de Biotecnología, and Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas,5 Madrid, Spain

Received 31 August 2009/Returned for modification 20 September 2009/Accepted 14 January 2010

Innate immunity to Candida albicans depends upon the recognition of molecular patterns on the fungal cell wall. However, the masking of major components such as β-glucan seems to be a mechanism that fungi have evolved to avoid immune cell recognition through the dectin-1 receptor. Although the role of C. albicans mitogen-activated protein kinase (MAPK) pathways as virulence determinants has been established previously with animal models, the mechanism involved in this behavior is largely unknown. In this study we demonstrate that a disruption of the C. albicans extracellular signal-regulated kinase (ERK)-like 1 (CEK1)-mediated MAPK pathway causes enhanced cell wall β-glucan exposure, triggering immune responses more efficiently than the wild type, as measured by dectin-1-mediated specific binding and human dendritic cell (hDC)- and macrophage-mediated phagocytosis, killing, and activation of intracellular signaling pathways. At the molecular level, the disruption of CEK1 resulted in altered spleen tyrosine kinase (Syk), Raf-1, and ERK1/2 activations together with IκB degradation on hDCs and increased dectin-1-dependent activator protein 1 (AP-1) activation on transfected cells. In addition, concurring with these altered pathways, we detected increased reactive oxygen species production and cytokine secretion. In conclusion, the CEK1-mediated MAPK pathway is involved in β-glucan exposure in a fungal pathogen, hence influencing dectin-1-dependent immune cell recognition, thus establishing this fungal intracellular signaling route as a promising novel therapeutic target.

Candida albicans is an opportunistic fungal pathogen that lives as a commensal on mucosal surfaces. In immunocompromised individuals this microorganism can behave as a pathogen, causing localized or disseminated candidiasis (41, 48). C. albicans is a polymorphic fungus able to change from the unicellular (yeast) to the mycelial (filamentous) form of growth, a process called dimorphic transition (3). Adaptation to a changing environment and coping with host defenses are essential for pathogen survival, and fungal mitogen-activated protein kinase (MAPK)-mediated signal transduction pathways are important for performing this function (45). A disruption of the CEK1-mediated pathway by the deletion of the CEK1 (Candida albicans extracellular signal-regulated kinase [ERK]-like 1) MAPK or the upstream HST7 MAPK kinase (MAPKK) genes causes defects in invasive growth (32), and cek1 mutants are less virulent in some animal models of candidiasis (10, 26). This route has also been related to cell wall biogenesis, as cek1 mutants displayed hypersensitivity to agents disturbing the cell wall (15, 47). However, the molecular mechanisms mediating these effects are not clarified.

The C. albicans cell wall is a complex dynamic structure based on a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin and an outer layer or matrix composed mainly of mannose-glycosylated proteins (42). The fungal cell wall surface represents the interface between the host and the infective pathogen. It is a valuable therapeutic target, as its highly conserved pathogen-associated molecular patterns (PAMPs) are recognized by different pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) (34) and C-type lectins (4, 23). Recognition by these PRRs mediates microbial uptake and killing as well as antigen presentation and the production of proinflammatory cytokines (48).

Dectin-1 is a C-type lectin receptor (28), expressed predominantly by myeloid cells, that specifically binds β-(1,3)-glucan (5, 7), a potent proinflammatory molecule that is normally hidden by the mannoprotein coat presumably to allow fungal escape from immune cell recognition (20, 58). As the main nonopsonic receptor involved in fungal uptake (27), dectin-1 ligation initiates intracellular signaling through the Syk- and CARD9-dependent pathways (25, 57), triggering different protective responses (22, 33, 36, 44). A recent study demonstrated that dectin-1 also signals through Raf-1 and that Syk- and Raf-1-dependent pathways converge at the level of NF-κB activation to control adaptive immunity to fungi (23). However, the generation of protective cytokine responses appears to require the simultaneous activation of TLR2 (6, 19). Although the role of dectin-1 in antifungal immunity in vivo is still controversial (50, 54), there are strong evidences supporting its involvement in the control of C. albicans infection (43). Most of the in vitro studies of dectin-1 engagement have used iso-
lated fungal components, particulate cell wall extracts (zymosan), or heat-killed cells as stimuli, which could not reflect the true complexity of the response to intact live fungi. Moreover, little is known about the relevance of β-glucan recognition by dectin-1 for the activation of the host defense in primary human cells, especially using whole live yeast cells. In this study, we show for the first time that a disruption of the CEK1-mediated MAPK pathway leads to altered β-glucan exposure, triggering enhanced dectin-1-mediated immune responses and further supporting a relevant role for this pathway in C. albicans immune evasion mechanism.

**MATERIALS AND METHODS**

*Candida albicans* strains and growth conditions. Unless otherwise stated, cek1, hst7, and cek2 indicate homologous Ura+ strains CK43B-16, CDEH32 (32), and BEC73 (15, 38), respectively, while CAF2 (16) and RM100 (1) were used as wild-type (wt) strains. Yeast cells were grown inYPD rich medium (2% glucose, 2% peptone, 1% yeast extract, 2% agar [if required]) at 30°C, and stationary-phase *Candida* cells were harvested by centrifugation and washed in PBS, and pellets were exposed to single-dose UV radiation (1.2 J/m²) for 1 h. Pellets were embedded in Durcupan resin. Ultrathin sections were viewed with a transmission electron microscope, and the images were recorded with a Show Scan camera (Leica Microsystems). The phagocytic index was assessed by using a Zeiss EM 900 transmission microscope, and the images were recorded with a Show Scan charge-coupled-device (CCD) camera (TRS).

**Generation of stable dectin-1 transfectants in K562 cells.** The dectin-1–Flag cDNA was obtained by reverse transcription (RT)-PCR amplification from peripheral blood mononuclear cell (PBMC) total RNA with specific primers for the full coding region of human dectin-1 plus the Flag epitope tag (underlined in the primer sequence) on the COOH terminus: sense primer 5'-tgctctagggctttgccgtgtcctggctag-3' and antisense primer 5'-ctcttgtcagctggtagaaccac-3'. The resulting pCDNA3.1-V5-His TOPO TA expression kit (Invitrogen). Plasmid pCDNA3.1-V5-Flag-Dectin-1-Flag was transfected into K562 cells with Cell Line Nucleofector Kit V (Amza Biosystems), and cells were selected by using neomycin (0.8 mg/ml). K562-dectin-1-Flag cells were isolated by using a FACsAria cell sorter (BD-Biosciences) after staining with anti-Flag monoclonal antibody (Mab) (Sigma-Aldrich).

**Generation of hMΦs and hDCs.** Human PBMCs were isolated from healthy donor buffy coats and purified as previously described (11, 51). Briefly, monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec). Growth and harvested by centrifugation and washed in PBS, and pellets were prefixed in a glutaraldehyde fixative (3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 h. Next, a mix with an agar solution (1.5%) was carried out, followed by a new glutaraldehyde fixation step for 30 min. After extensive washing with the phosphate buffer, postfixation was carried out with 1% osmium tetroxide for 1 h. Pellets were embedded in Durcupan resin. Ultrathin sections were stained with 2% uranyl acetate. Samples were imaged with a Zeiss EM 900 transmission microscope, and the images were recorded with a Show Scan charge-coupled-device (CCD) camera (TRS).

**Measurement of ROS.** Generation of hMΦs and hDCs. Human PBMCs were isolated from healthy donor buffy coats and purified as previously described (11, 51). Briefly, monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec) and cultured in complete Dulbecco's modified Eagle's medium (DMEM) (10% heat-inactivated fetal calf serum [FCS], 1% penicillin-streptomycin, 4 mM glucose, and 20 mM HEPES) with the addition of fluorescein isothiocyanate (FITC)-labeled C. albicans (10 yeast cells/1 ml). Fungicidal assays with hDCs and hMΦs. Killing assays were performed as previously described (12). Briefly, yeast cells and hDCs or hMΦs were incoculated (1 yeast cell:20 phagocytes) for 4 h. After phagocytic lysis with water, serial dilutions were spread over YPD agar plates for determinations of CFU after 24 h of incubation (37°C). The killing percentage for each strain was expressed as the percent reduction of CFU from hDC- or hMΦ-yeast cocultures versus simultaneous co-culturing of yeast cells without phagocytes.

**Western blot analysis.** Overnight culture in serum-reduced medium, cells were treated with lysis buffer (40 mM Tris [pH 7.6], 150 mM NaCl, 1% NP-40, 0.1% SDS, 10 g/ml phenylmethylsulfonyl fluoride [PMSF], 100 mM sodium fluoride [NaF], 10 mM sodium pyrophosphate, 10 mM sodium vanadate, 10 mM sodium orthovanadate, 10 mM sodium molybdic acid, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate). Western blot analysis was performed using antibodies specific for anti-phospho-p44/42 MAPK (Cell Signalling), for anti-phospho-Syk (Calbiochem)/anti-Syk MAb (Upstate), and antisense primer 5'-tgctctagggctttgccgtgtcctggctag-3' and antisense primer 5'-ctcttgtcagctggtagaaccac-3'. The resulting pCDNA3.1-V5-His TOPO TA expression kit (Invitrogen). Plasmid pCDNA3.1-V5-Flag-Dectin-1-Flag was transfected into K562 cells with Cell Line Nucleofector Kit V (Amza Biosystems), and cells were selected by using neomycin (0.8 mg/ml). K562-dectin-1-Flag cells were isolated by using a FACsAria cell sorter (BD-Biosciences) after staining with anti-Flag monoclonal antibody (Mab) (Sigma-Aldrich).
Cold PBS, and ROS production was examined by flow cytometry. Data are shown as the mean fluorescence intensities (MFIs) given by H$_2$DCFDA in the presence of the different C. albicans strains relative to the MFI in the presence of PBS.

**Stimulation of cytokine production in hDCs.** A total of $0.8 \times 10^6$ hDCs were cocultured with zymosan (200 μg/ml; Sigma-Aldrich) and UV-inactivated or heat-killed C. albicans strains (50 yeast cells:1 cell). For blocking, hDCs were preincubated (30 min at 37°C) with anti-human dectin-1 MAb (MGD3; 5 μg/ml) or a functional-grade purified IgG2a isotype control (eBiosciences) before coculture with UV-inactivated strains (10 yeast cells:1 cell). Controls included cells cultured in medium alone. Supernatants were analyzed for IL-10 and tumor necrosis factor alpha (TNF-α) by using commercial ELISA pair sets (R&D and C. A. Biosystems). Dectin-1 cell expression was determined with MAb MGD3 (for immunofluorescence) secondary labeled antibody (Dako and Molecular Probes, respectively). Dectin-1 cell expression was determined with MAb MGD3 for immunofluorescence assays and (Jackson ImmunoResearch) followed by FITC (for cytometry) or Alexa-488 (for immunofluorescence measurement) secondary labeled antibody (Dako and Molecular Probes, respectively). Dectin-1 cell expression was determined with MAb MGD3 followed by an FITC-labeled secondary Ab. Incubations were done at 4°C in staining PBS. Flow cytometry analyses were performed with a FACSCalibur flow cytometer using CellQuestPro software (BD-Biosciences), and immunofluorescence preparations were analyzed by confocal microscopy (described above) or a conventional Leica DMR photomicroscope with QFISH software (Leica Microsystems).

**Statistical analyses.** The differences between groups were analyzed by the Mann-Whitney U test. The levels of significance among groups were set at a $P$ value of <0.05, and a $P$ value of <0.001. Unless otherwise stated, all experiments were performed at least five times, and the data are given as mean values ± SD.

## RESULTS

**Disruption of the C. albicans CEK1-mediated MAPK pathway alters cell wall morphology.** In the fungal pathogen C. albicans, MAPK pathways are mechanisms by which different types of stress (oxidative, temperature, pH, and others) are sensed and an appropriate response is developed (Fig. 1A). The cek1 mutant is defective in the Cek1 MAPK, which participates in cell wall construction (15) and becomes activated during growth-associated cell wall remodeling (47). In order to identify cell wall alterations on the cek1 mutant compared to wild-type (wt) strain CAF2, transmission electron microscopy (TEM) analysis was initially performed. Although cell wall thicknesses were not significantly different between them (data not shown), TEM micrographs revealed a less-electron-dense cell wall on the cek1 mutant compared to wt strain CAF2 (Fig. 1B and C). This differential cell wall density on the cek1 mutant could account for its hypersensitivity to agents disturbing the cell wall (15) and might modify fungal detection by human immune cells.

**The C. albicans CEK1-mediated MAPK pathway controls β-glucan exposure and dectin-1-mediated fungal recognition.** Considering the C. albicans cell wall structure, we hypothesized that the less electron-dense cell wall of cek1 mutants on TEM micrographs could reflect enhanced β-glucan exposure. Flow cytometry measurements revealed that β-glucan exposure is significantly higher in live cek1 deletion mutants than in wt CAF2 yeast cells (Fig. 2A). Moreover, the recognition of the cek1 mutant by soluble Fc-tagged dectin-1 recombinant protein (sDectinFc) was also considerably elevated (Fig. 2A), thus demonstrating that a CEK1 mutation leads to enhanced β-glucan exposure and dectin-1 recognition. Assays with heat-killed (HK) fungi, which show increased β-glucan exposure at the cell surface (20, 58), were used as positive controls (Fig. 2A). Confocal microscopy analysis indicated that β-glucan was found over the entire surface of the mutant, whereas wt fungi showed a restricted pattern corresponding to the mother-daughter septal regions (20) (Fig. 2B). A similarly increased level of β-glucan staining was seen in the upstream MAPK K Hst7 mutant (hst7), further confirming the implication of the CEK1-mediated MAPK route for β-glucan exposure (see Fig. S1A and S1B in the supplemental material). A tridimensional analysis of the yeast cell wall revealed an even staining over the

---

**FIG. 1.** *Candida albicans*-MAPK signal transduction pathways and cell wall morphology of C. albicans wild-type CAF2 and the cek1 deletion mutant. (A) The main elements of MAPK signal transduction pathways in C. albicans are schematized, and the physiological function of each pathway is indicated. The CEK1-mediated MAPK pathway is highlighted. (B and C) Transmission electron micrographs of the cell wall of wt strain CAF2 (B) and the cek1 deletion mutant (C), which lacks Cek1 MAPK. Scale bar, 100 nm.
entire surface of the cek1 mutant when β-glucan MAb was used, whereas a patched recognition pattern was detected with sDectinFc (Fig. 2C). In addition to the above-described delocalized pattern, bud scars were strongly stained, showing that β-glucan is exposed in these surface structures (Fig. 2C). For wt fungi, the tridimensional reconstruction confirmed the restricted pattern shown in Fig. 2B. Finally, in order to analyze whether the mannoprotein content was also different on the surface of the C. albicans strains used, concanavalin A (ConA) staining of both the wt and the cek1 mutant was performed, and no significant differences were found (see Fig. S2A and S2B in the supplemental material), further supporting the specific increase of β-glucan exposure on cek1 mutants. Taken together, these data demonstrate that the disruption of the CEK1-mediated MAPK pathway leads to enhanced β-glucan exposure, causing increased dectin-1-mediated recognition.

Dectin-1-mediated binding to C. albicans is increased specifically in cek1 mutants. To extend the above-described delocalized pattern, bud scars were strongly stained, showing that β-glucan is exposed in these surface structures (Fig. 2C). For wt fungi, the tridimensional reconstruction confirmed the restricted pattern shown in Fig. 2B. Finally, in order to analyze whether the mannoprotein content was also different on the surface of the C. albicans strains used, concanavalin A (ConA) staining of both the wt and the cek1 mutant was performed, and no significant differences were found (see Fig. S2A and S2B in the supplemental material), further supporting the specific increase of β-glucan exposure on cek1 mutants. Taken together, these data demonstrate that the disruption of the CEK1-mediated MAPK pathway leads to enhanced β-glucan exposure, causing increased dectin-1-mediated recognition.

Deletion of CEK1 augments phagocytosis by and susceptibility to human phagocytes. Macrophages (Mφs) and dendritic cells (DCs) are strategically located at the sites of Candida entry (mucosal surfaces and skin), being essential to initiate antifungal innate immune responses. Phagocytosis facilitates the removal and killing of pathogens and primes the adaptive

FIG. 2. Deletion of the CEK1-mediated MAPK pathway results in cell wall β-glucan exposure and increased dectin-1 recognition. (A) Representative flow cytometry analysis of β-glucan exposure on live C. albicans wt CAF2 and cek1 deletion mutants. Light-gray-filled histograms correspond to control antibody (Ab), dark-gray-filled histograms correspond to anti-β-glucan MAb or soluble dectin-1–Fc (sDectinFc), and the empty histogram represents heat-killed positive-control yeast cells. Statistical analysis of these data is shown below and represents the mean fluorescent intensity (MFI) ± SD (after the subtraction of control Ab MFI). **, P < 0.001. (B) Confocal immunofluorescence analysis of individual yeast cells showing representative differential interference contrast (DIC) (Nomarski) microscopy and fluorescence images overlaid. Fungi were stained alternatively with anti-β-glucan MAb or sDectinFc followed by Alexa-488-labeled secondary Abs. (C) Confocal immunofluorescence analysis of individual yeast cells stained as described above (B) showing a tridimensional reconstruction of z-stack fluorescence images. Arrows indicate stronger-stained patches corresponding to bud scars. For CAF2 yeast cells, DIC and fluorescence image overlays are also shown.
immune response (29). To evaluate the physiological relevance of cek1-dependent enhanced β-glucan exposure, the phagocytosis of both strains (the cek1 mutant and wt CAF2) by human monocyte-derived DCs and MΦs (hDCs and hMΦs, respectively) was analyzed, and the phagocytic index was determined through immunomicroscopical procedures to discriminate between bound and internalized fungi (Fig. 4A and B). For both types of phagocytes, the phagocytic index of cek1 was approximately twice that of wt CAF2, with hDCs exhibiting a higher phagocytic index than hMΦs (Fig. 4C and D). Strikingly, the anti-dectin-1 MAb (MGD3) inhibited phagocytosis by both hDCs and hMΦs to a similar extent (Fig. 4C and D), although it was slightly more effective in hDCs. Moreover, it seems that MGD3 pretreatment did not affect CAF2 uptake, while it specifically inhibited cek1 phagocytosis. These data demonstrate a critical role for dectin-1 in cek1 phagocytosis by both hDCs and hMΦs. Regarding pathogen viability after exposure to immune cells, killing assays revealed that the cek1 mutant strain was up to three times more susceptible to hDCs than the corresponding wt strain (Fig. 4E). In contrast, both strains are equally susceptible to hMΦ killing (Fig. 4F), in agreement with previously reported results (2). Altogether, this set of data indicates that the deletion of CEK1 increases β-glucan exposure on the Candida cell wall, giving a boost to dectin-1-dependent fungal recognition and phagocytosis by both hDCs and hMΦs and increasing killing by hDCs.

The C. albicans cek1 mutant promotes phosphorylation of Syk, Raf-1, and ERK kinases inducing IκB degradation in hDCs and triggers activator protein 1 (AP-1) activation through dectin-1. To determine the molecular basis for the differential handling of both strains by human mononuclear phagocytes, the dectin-1-dependent signaling pathways were
evaluated with hDCs exposed to wt and mutant yeast cells. As shown in Fig. 5A, Syk phosphorylation was observed only after stimulation with the cek1 mutant. Moreover, and unlike wt yeast cells, which stimulated early but short ERK phosphorylation, the cek1 mutant triggered stronger and sustained ERK activation (Fig. 5A). Furthermore, and in agreement with data from a recent report on dectin-1-dependent Raf-1 activation (23), the cek1 mutant activated the Raf-1-dependent signaling pathway more strongly than did the wt. Dectin-1–C. albicans engagement ultimately activates the transcription factor NF-κB through Syk- and Raf-1-dependent signaling pathways. To indirectly analyze the activation levels of NF-κB, we thus...
studied IxB degradation on hDCs exposed to different strains. Only cek1 yeast cells were able to induce a late (30 min) degradation of IxB, indirectly indicating NF-kB activation.

Finally, previous studies reporting AP-1 activation via the dectin-1/Syk pathway during fungal infection (18, 56) led us to analyze the effect of cek1 on the dectin-1-dependent activation of AP-1. To avoid a yeast-to-hypha transition during the experiment and to minimize cell killing, we used single-dose UV-inactivated fungi (sufficient to inactivate the fungus while avoiding cell wall disruption and consequent β-glucan unmasking [see Fig. S4 in the supplemental material]). Luciferase reporter assays with transfected HEK293T cells transiently transfected with the indicated plasmids after incubation with zymosan (ZYM) or C. albicans UV-inactivated yeast cells. Luciferase activity was calculated as firefly/Renilla luciferase activities and normalized versus the control (untreated). Bars represent relative light units (RLU) as means ± SD for five independent experiments, each carried out in duplicate. Statistical analyses compared the luciferase activity induction of HEK293T–dectin-1-transfected cells exposed to the wt versus the mutant strain. *, P < 0.05. The inset shows the flow cytometry expression profile of HEK293T–dectin-1-transfected cells (filled histogram, control Ab; empty histogram, dectin-1 staining).

Deletion of the CEK1 gene leads to an augmented respiratory burst and enhanced dectin-1-dependent cytokine synthesis. In addition to binding, phagocytosis, killing, and intracellular signaling, the interaction of C. albicans with phagocytes ultimately leads to respiratory burst activation and cytokine release (48). Thus, we tested the abilities of the cek1 mutant and the wt strain to induce the phagocyte respiratory burst in hMφs and hDCs. The cek1 mutant promoted a slight but significant increase in ROS secretion compared to that of the wt (Fig. 6A and B) in both types of cells. In addition, and in accordance with the ERK activation observed after intracellular ROS production (17), a stronger phosphorylation of ERK in RAW 264.7 Mφ cells exposed to the cek1 mutant was observed (data not shown).

Finally, since the activation of the dectin-1-dependent Syk pathway on hDCs leads to ERK phosphorylation (Fig. 5A) and the production of cytokines (13, 53), the effect of the cek1 mutant on hDC cytokine synthesis was evaluated. UV-inactivated fungi induced lower levels of TNF-α secretion than heat-killed fungi (Fig. 6C), concurring with previous observations of heat treatment-induced cell wall disturbances (20, 22, 58). Nonetheless, when the C. albicans cell wall was undisturbed (single-dose UV-treatment [see Fig. S4 in the supplemental material]), the cek1 mutant induced a slight but significant increase in TNF-α secretion compared to that produced by wt yeast cells (Fig. 6C). This cytokine secretion was dectin-1 dependent, as it was blocked by anti-human dectin-1 MAb MGD3 (Fig. 6D). In addition, we also observed that Cek1-deficient fungi induced a later and higher level of dectin-1-mediated IL-10 production than wt yeast (Fig. 6D), and this effect was significantly inhibited in the presence of MAb MGD3. Therefore, the deletion of the Cek1 kinase in C. albi-
C. albicans results in an enhancement of all the known cellular responses previously ascribed to dectin-1 ligation, establishing this signaling route as a potential target for the modulation of antifungal immune responses.

**DISCUSSION**

*Candida albicans* is the most common fungus causing nosocomial infections and remains a major diagnostic and therapeutic challenge to the clinician (49). As the unmasking of β-glucan increases the recognition of this fungus by innate immune cells (22, 58), the identification of genes involved in this process has attracted research interest for the development of new antifungal therapies. Here we report the involvement of a MAPK signaling pathway in β-glucan masking in a pathogenic fungus. We demonstrated that a Cek1 deficiency enhanced β-glucan exposure on the *C. albicans* cell wall, leading to greater innate immune responses triggered through dectin-1.

In the present study we detected β-glucan exposure over the entire cell wall surface on *cek1* and *hst7* deletion mutants, whereas on wt yeast cells, β-glucan was found only in discrete patches. The unmasking of β-glucan increased dectin-1-mediated recognition, uptake, killing, and signaling by phagocytic cells. In this regard, the activations of Syk-, Raf-1-, and ERK-dependent pathways along with IκB degradation in hDCs were enhanced, as was dectin-1-dependent AP-1 activation in dectin-1-transfected cells. As a consequence of this signaling activation, we detected enhanced ROS production and cytokine secretion. It is noteworthy that these data suggested that this enhanced dectin-1-mediated immune response could explain the attenuated virulence of *cek1* mutants in animal models of disseminated or localized candidiasis (10, 26). On the other hand, it was previously described that under certain conditions, the Cek1 MAPK participates in filamentation and invasion (32). This route is also involved in cell wall biogenesis, as either *cek1* mutants or mutants defective in their phosphorylation (*hst7* or *sho1*) are sensitive to compounds such as Congo red,
could result in additional phenotypes that cannot be excluded from research into the specific regulation of yeast-killing mechanisms elicited following the recognition of dectin-1. The mosaic of PRRs that is expressed by each of hDCs. In addition, MΦ/hM9021 the specifically enhanced attenuated cytocidal function may not be sufficient to initiate a significant. This lower level of surface expression together with the lower than that on hDCs, since MGD3 blocking is less significant. This lower level of surface expression together with the attenuated cytocidal function may not be sufficient to initiate the specifically enhanced cek1 killing mechanisms observed for hDCs. In addition, MΦs express higher levels of TLRs than DCs (39) that could mask the specific cek1 killing mediated by dectin-1. The mosaic of PRRs that is expressed by each of these cell types ultimately determines the type of response elicited following the recognition of C. albicans. However, further research into the specific regulation of yeast-killing mechanisms will shed light on this. Nonetheless, a CEK1 disruption could result in additional phenotypes that cannot be excluded as a cause of their virulence defects. These data allow us to speculate that dectin-1−/− mice should be more susceptible than wt mice to infection with Cek1-deficient strains. However, the outcome of experimental infections is influenced by several mechanisms, including PRR recognition and cytokine production, as well as other factors such as adherence to host cells and the growth rate of the mutants. Further in vivo studies to evaluate the relevance of β-glucan unmasking on the immune response would thus be of interest. Regarding this issue, a recent study by Wheeler et al. (59) showed that β-glucan is progressively unmasked during infection, further supporting a major role of dectin-1 in protective antifungal immunity.

Dectin-1 engagement by the cell wall extract zymosan in DCs triggers Syk and ERK pathway activation (13, 44). Regarding Syk phosphorylation, we observed activation only after the stimulation of hDCs with the cek1 mutant. Additionally, cek1-induced ERK activation was sustained in comparison to that of wt yeast. These data concur with previous observations showing ERK phosphorylation coupled to dectin-1/Syk signaling (35, 53). We also showed that the cek1 mutant induced the Raf-1 signaling pathway (23) more strongly than the wt. Although Syk activation was induced exclusively through dectin-1, Raf-1 activation is induced through both dectin-1 and DC-SIGN (24), explaining the baseline activation of hDCs exposed to wt CAF2 cells. Finally, previous studies showed that dectin-1 activates NF-κB (19, 25) and AP-1 (56) via Syk. It thus appears that the enhanced β-glucan exposure in the cek1 mutant induces increased dectin-1 recognition, leading to Syk/ERK/Raf-1 phosphorylation, which triggers NF-κB and AP-1 activation and ultimately leads to ROS production and cytokine synthesis. In this regard, the cek1 mutant elicited slight but significant TNF-α secretion. In addition, significant IL-10 production from hDCs was detected, which, at later stages of the infection, may be beneficial to resolving an inflammatory process (13, 48). The weak cytokine response elicited by C. albicans wt cells may prevent the recruitment of effector cells and the elimination of the pathogen, explaining its persistence as a commensal. Nonetheless, the biological relevance of the slight increase in cytokine synthesis induced by the cek1 mutant is unclear, and this increase alone might not account for the loss of virulence, while the enhanced phagocytosis and killing observed for hDCs may help the transition from commensalism to infection.

During C. albicans infection, both yeast and filamentous forms can be found in infected tissues, and DCs discriminate between them, eliciting a protective response against yeast and tolerance to hyphae through different PRRs (14). An integrated model of fungal recognition has been proposed, in which immune sensing of C. albicans requires the cooperative recognition of mannans and glucans by PRRs (39). The outcome of an immune response to C. albicans thus depends on the balance of the signals generated through TLR2, TLR4, dectin-1, MR, and DC-SIGN, among others, each of which recognizes a different PAMP of the fungal cell wall. Both TLR2 and TLR4 collaborate with dectin-1 to induce an inflammatory response (6, 12, 19, 40). Herein we showed that dectin-1-specific activation by C. albicans occurs only when β-glucan is exposed, concurring with data from previous studies (20, 59). The enhanced uptake, killing, and cytokine synthesis observed for hDCs exposed to the cek1 mutant were thus inhibited by dectin-1 MAb MGD3, suggesting a dectin-1-dependent elicitation. Nonetheless, we cannot exclude the participation of other PRRs in this process (8, 9, 21, 24). Further studies are needed to evaluate the complex cross talk between dectin-1 and other PRRs in immunity to C. albicans, mainly in the context of the in vivo recognition of intact fungi by primary human immune cells. In this regard, we observed that responses elicited by live or UV-inactivated fungi were weaker than those triggered by heat-killed yeast (Fig. 6B), indicating the need for studies using whole live or suitable UV-inactivated C. albicans yeasts to avoid misleading conclusions (see Fig. S4 in the supplemental material). Moreover, conventionally used isolated fungal components or zymosan could not reflect the true complexity of the response against intact live fungi. Therefore, the preservation of cell wall structure is of outstanding importance in elucidating the immune cell-fungus interaction.

Finally, as we have demonstrated that increased cek1 mutant β-glucan exposure led to enhanced innate immune activation, the CEK1-mediated MAPK pathway can be proposed as a valuable antifungal target. Moreover, drug-induced β-glucan exposure could target fungi for recognition by natural anti-β-glucan antibodies, which are detected in patients with progressive fungal infection (42, 55), rendering the pathogen more susceptible to the host immune system.

In conclusion, the present study demonstrated that the CEK1-mediated MAPK pathway has a key role in β-glucan masking in C. albicans and that, given the high degree of conservation in MAPK pathways, this phenomenon may be a general mechanism of fungi to evade host recognition. Addi-
tionally, our study highlights the value of fungal MAPK pathways as potential therapeutic targets in modulating the host immune response to a pathogen.

ACKNOWLEDGMENTS

We thank F. Molina for help in using the confocal microscope, C. Santiago for assistance with confocal microscopy, F. Schroer, M. Llorente for help with hybridoma production, M. Martin for biosensor assays, J. Gonzalez for TEM micrographs, and S. Chamorro, I. Olazabal, and P. Majano for their critical reading of the manuscript and helpful suggestions.

This work was partially supported by Ministerio de Ciencia e Innovacion (MICINN) grants PI05/1999 and PI08/1772, Fundación de Investigación Médica Mutua Madrileña, to E.F.-R.; Instituto de Salud Carlos III-FEDER, Spanish Network for the Research in Infectious Diseases, grant REIPI RD06/008 to E.-F.R. and A.-L.C.; grants BIO2009-07788 and GEN2006-27775-C1-1-1PAT to J.P. and grant BFU2005-05972 to J.M.C. from the MICINN; and Consejo de Investigaciones Científicas grant CSIC-2009201016 to L.K. (Protein Tools Unit). M.G.-D. was supported by the Consejería de Educación de Investigaciones Científicas grant CSIC-2009201016 to L.K. (Protein Tools Unit). M.G.-D. was supported by the Fundación de Investigación Biómédica of the Hospital Universitario de la Princesa.

REFERENCES