The pathobiology of *Paracoccidioides brasiliensis*

M. Ines Borges-Walmsley, Daliang Chen, Xinhua Shu and Adrian R. Walmsley

Paracoccidioides brasiliensis **causes one of the most prevalent systemic mycoses in Latin America – paracoccidioidomycosis. It is a dimorphic fungus that undergoes a complex transformation *in vivo*, with mycelia in the environment producing conidia, which probably act as infectious propagules upon inhalation into the lungs, where they transform to the pathogenic yeast form. This transition is readily induced *in vitro* by temperature changes, resulting in modulation of the composition of the cell wall. Notably, the polymer linkages change from β-glucan to α-glucan, possibly to avoid β-glucan triggering the inflammatory response. Mammalian oestrogens inhibit this transition, giving rise to a higher incidence of disease in males. Furthermore, the susceptibility of individuals to paracoccidioidomycosis has a genetic basis, which results in a depressed cellular immune response in susceptible patients; resistance is conferred by cytokine-stimulated granuloma formation and nitric oxide production. The latency period and persistence of the disease and the apparent lack of efficacy of humoral immunity are consistent with *P. brasiliensis* existing as a facultative intracellular pathogen.**

M. Ines Borges-Walmsley
Daliang Chen
Xinhua Shu
Adrian R. Walmsley*
Division of Infection and Immunity,
Robertson Building,
Institute of Biomedical and Life Sciences,
University of Glasgow,
Glasgow, UK G11 6NU.
*e-mail:
a.walmsley@bio.gla.ac.uk

Invasive fungal diseases pose a serious and growing health problem and are a major cause of death worldwide. Although fungal pathogens are increasingly becoming the focus of medical research as new prophylactic measures are sought, this research has largely focussed on those fungal pathogens that cause diseases in the developed world, such as *Candida albicans*, whereas other fungal pathogens that predominate in developing countries have received much less attention. One such fungal pathogen is *Paracoccidioides brasiliensis*, the cause of paracoccidioidomycosis, which is one of the most prevalent human systemic mycoses in Latin America [1]. It is estimated that throughout the endemic region, in which ~90 million people live, as many as ten million individuals could be infected with *P. brasiliensis* [2].

P. brasiliensis is a dimorphic fungus that undergoes a complex transformation *in vivo* [1]. The ploidy of *P. brasiliensis* is not known with certainty but the available data suggest that it is diploid. Four or five chromosomes, varying in size from 2–10 Mb, have been identified and the genome size is estimated to be 23–30 Mb, indicating the presence of 10 000–15 000 genes [3,4]. Conidia, produced by saprobic mycelia in the environment, probably act as the infectious propagules and are inhaled into the lungs where transformation to the pathogenic yeast form occurs [1,5]. Infection can give rise to either an asymptomatic condition or to active disease and initially causes pulmonary lesions in the lungs but can subsequently disseminate to other organs and tissues. Paracoccidioidomycosis occurs mainly in immunocompetent individuals and there is a gender bias towards males. The disease often resembles tuberculosis, with which it occurs concurrently in about 10% of cases.

Paracoccidioidomycosis: the disease and infection

There are two main clinical forms of paracoccidioidomycosis: the acute or sub-acute form (juvenile type) and the chronic form (adult type), although both the clinical presentation and the course of disease vary from patient to patient [1,6]. Both forms are associated with extensive sequelae – mediated by systemic infections – including lesions and an abnormal cell-mediated immune response. The adult type of paracoccidioidomycosis, which accounts for >90% of cases, mostly in adult males, progresses slowly and can take months to years to develop fully. Adult paracoccidioidomycosis primarily afflicts the lungs, leading to significant morbidity due to gross impairment of lung function. Subsequently, the disease can disseminate to other organs and tissues, forming secondary lesions in the mucous membranes, skin, lymph nodes and adrenal glands. By contrast, the juvenile type of paracoccidioidomycosis, which develops within weeks to months, is more severe, leading to significant rates of mortality due primarily to reticuloendothelial system organ hypertrophy. Regardless of the organ involved, paracoccidioidomycosis usually heals by fibrosis, with the formation of fibrotic sequelae, which can permanently interfere with the well-being of the patient.

The treatment of paracoccidioidomycosis is usually prolonged, with many patients receiving therapy for between one and two years; in the absence of drug therapy the disease is usually fatal. Currently, ketoconazole and itraconazole are the drugs of choice over sulfonamides and amphotericin B, for which patient-relapse rates are high [7]. Although azole and other drugs can arrest the progression of paracoccidioidomycosis, the fibrotic sequelae persist, probably constituting a source of *P. brasiliensis* that could lead to a relapse in the disease following termination of treatment. Indeed, a feature of *P. brasiliensis* is its ability to initiate infection after a prolonged period of dormancy. For example, it is not uncommon for patients to fall ill more than a decade after leaving an endemic region [1]. The molecular mechanisms underpinning these processes in *P. brasiliensis* appear to be analogous to those seen in

http://tim.trends.com 0966-842X/02/$ – see front matter PII: S0966-842X(01)02292-2

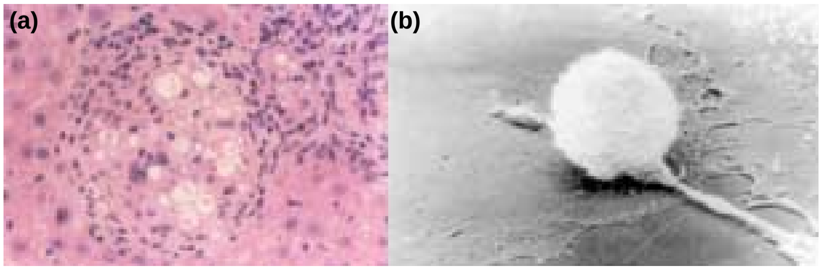


Fig. 1. (a) A granuloma produced in the liver of wild-type mice following infection with *Paracoccidioides brasiliensis*, and (b) scanning electron micrograph of cultured epithelial (Vero) cells after incubation with *P. brasiliensis*, showing yeast cells adhering to the surface of the epithelial cells through a narrow tube. Images (a) and (b) kindly provided by Vera Calich at the Universidade de São Paulo (USP) and Maria Giannini at the Universidade Estadual Paulista (UNESP), respectively. (b) Reproduced with permission from [47].

Mycobacterium tuberculosis, where gradual oxygen depletion induces a downshift to a state in which the organism can survive micro-aerophillic and anaerobic conditions, such as those found in granulomas or lesions of the lung [8,9].

Regulation of the immune response

The clinical form of the disease is contingent upon the patients' immune response. In contrast to patients with the mild, chronic form of paracoccidioidomycosis, patients with the severe, acute form of the disease often have a depressed cellular immune response [1,10,11]. Many individuals exposed to the organism develop an asymptomatic infection, indicating that they are resistant, whereas other individuals are highly susceptible to infection and develop overt paracoccidioidomycosis. A murine model of paracoccidioidomycosis has been developed that demonstrates differences in the susceptibility of inbred mice according to their genetic background: A/Sn mice are resistant to intraperitoneal infection with *P. brasiliensis* yeast cells whereas B/10.A mice are susceptible [12]. A drawback of this intraperitoneal model is that it does not mimic the putative infective propagules or the natural route of infection (i.e. intranasal conidia). However, pulmonary models using intranasal inoculation of B/10.A and A/Sn mice with yeast cells [12] or BALB/c mice with conidia [13] have also been developed, and are used extensively because they mimic the human disease in the formation of granulomas and the development of pulmonary fibrosis. No major differences have been noted in the immune response of mice infected via these different routes. These studies have led to the proposal that resistance has a genetic basis and is controlled by a single dominant autosomal gene, in analogy to the *Nramp1* gene, which is expressed in macrophages and, possibly by raising interferon (IFN)-γ levels, controls resistance to intracellular pathogens such as *Salmonella*, *Mycobacteria* and *Leishmania* in mice [14].

The main host defence against *P. brasiliensis* is a cell-mediated rather than humoral immune response. In patients with less severe disease, the host immune response induces dense, compact granulomas that are associated with low numbers of fungal cells; patients with severe disease frequently have loose granulomas that are associated with high numbers of viable budding yeast cells. Macrophages form the major cell defence against *P. brasiliensis*, ingesting the organism, which can undergo intracellular replication in the absence, but not the presence, of IFN-γ [15]. Polymorphonuclear leukocytes (PMNs) have also been shown to exert a fungistatic effect on *P. brasiliensis* yeast cells, which is enhanced by IFN-γ and granulocyte–macrophage colony-stimulating factor [16]. Similarly, infection of resistant and susceptible mice via the intraperitoneal or intranasal route is characterized by high and low activation status, respectively, of macrophages and PMNs [12] (Fig. 1a).

The major *P. brasiliensis* antigen is an extracellularly secreted 43-kDa glycoprotein termed gp43 [17]. When challenged with gp43, healthy individuals sensitized to *P. brasiliensis* produce substantial levels of IFN-γ, interleukin (IL)-2 and IL-10, reflecting a well-balanced, effective immune response [18]. By contrast, paracoccidioidomycosis patients produce low levels of IL-2, IFN-γ and tumour necrosis factor (TNF)-α but substantial amounts of IL-10 [18,19]. Furthermore, IFN-γ levels are restored in paracoccidioidomycosis patients following clinical remission [20]. This imbalance in cytokine secretion could downregulate the host immune response by decreasing the microbiocidal activity and antigen-presentation abilities of macrophages. BALB/c mice challenged with gp43 mount a Th1 immune response with secretion of IFN-γ and IL-2 [21]. The T-cell epitope in gp43 has been mapped to a 15-residue sequence, and immunization with the corresponding oligopeptide elicited a protective response against virulent *P. brasiliensis* [21]. Furthermore, DNA-based vaccination using the *gp43* gene elicited protective immunity against *P. brasiliensis* in BALB/c mice [22].

A greater insight into the immune response elicited by *P. brasiliensis* has come from the use of murine models [12]. Resistance in mice is linked to a preferential T-helper 1 (Th1) immune response, with elevated secretion of IFN-γ and IL-2, and susceptibility is associated with very low levels of these cytokines [23]. Both susceptible and resistant mice produced IL-10 throughout the course of the disease, which might downregulate the secretion of IFN-γ in susceptible mice, reducing fungicidal activity. The absence of IL-4 secretion early in the infection process makes it difficult to conclude susceptibility results from a typical Th2 response. The depletion of IFN-γ in both resistant and susceptible mice using monoclonal antibodies to IFN-γ exacerbates the pulmonary infection, with earlier dissemination of the fungus to other tissues [24]. Furthermore, IFN-γ-knockout mice are highly susceptible to paracoccidioidomycosis, produce loose granulomas and quickly succumb to the disease and its resulting mortality, owing to early fungal dissemination [25,26]. Recent studies indicate that IFN-γ activates macrophages to produce the reactive nitrogen intermediate nitric oxide (NO), which inhibits the transformation of *P. brasiliensis* conidia to yeast but probably has the secondary effect of causing the immunosuppression noted in both human and murine paracoccidioidomycosis [27,28]. In addition, macrophages appear to use an iron-restriction mechanism to inhibit the transformation of ingested conidia to yeast [29]. IFN-γ also stimulates *P. brasiliensis*-infected macrophages to secrete TNF-α, which is required for the persistence of well-formed granulomas [25]. Knockout mice lacking the p55 receptor for TNF-α are more susceptible to infection, with a resultant increase in the number of viable yeast cells and loss of granuloma formation [25,26]. This increase in susceptibility of the p55-receptor-knockout mice was associated with reduced NO production. Thus, both INF-γ and TNF-α apparently confer resistance to *P. brasiliensis* by stimulating granuloma formation and NO production, thus controlling infection. Recent studies with human monocytes indicate that the effects of IFN-γ and TNF-α are negatively regulated by prostaglandins, which are probably secreted by the host cells in response to the pathogen and could be involved in suppressing the immune response [30].

Although humoral immune responses are not thought to play a major role in defence, both patients suffering from acute paracoccidioidomycosis [10] and infected susceptible mice [12] produce large amounts of specific antibodies. Significant titres of these antibodies often persist after successful treatment of the disease, suggesting they have no protective role. However, the recent demonstration that both humans and mice produce large quantities of anti-idiotypic and anti-anti-idiotypic antibodies, which correlate with the severity of the disease, points to a more complex humoral immune response that awaits further elucidation [31].

Attention has also focussed on the ratio of $CD4^+$ to $CD8^+$ cells as lower ratios have been associated with impaired cell-mediated immunity, and these studies provide some evidence of a lower ratio in individuals suffering from paracoccidioidomycosis [18,19]. However, this finding seems to be at odds with the low prevalence of paracoccidioidomycosis in immunosuppressed patients (e.g. in AIDS patients, of which there are many in endemic regions) [32]. In resistant and susceptible mice, $CD8^+$ cells have also been shown by depletion with monoclonal antibodies to have a role in clearing fungal cells and controlling their dissemination, but not in the production of specific antibodies [33].

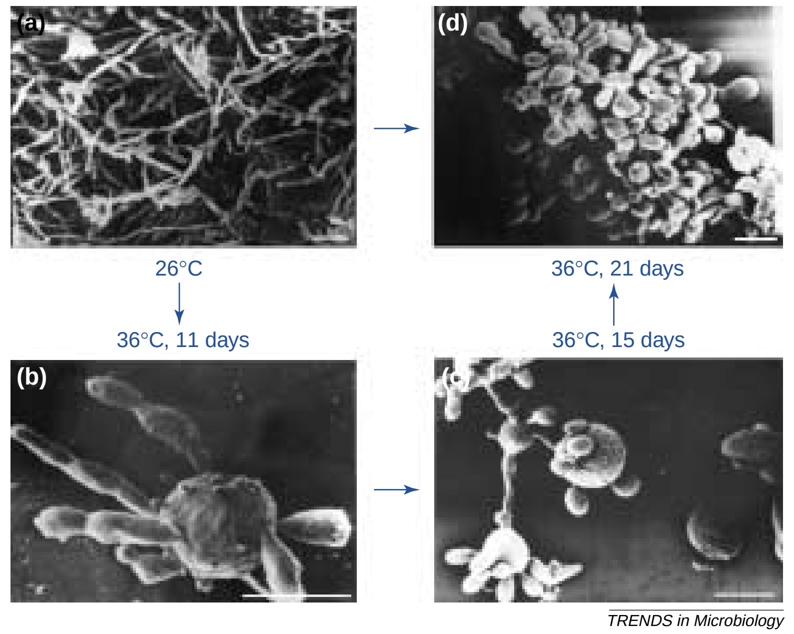
Paracoccidioidomycosis predominates in males

There is a pronounced sex bias in *P. brasiliensis* infection, with a ratio of male to female adult infections of up to 78:1 [34]. The greater incidence of clinical disease in males cannot be accounted for purely in terms of differential exposure because subclinical infection does not reveal this sex bias. The mechanism underlying this process is thought to involve hormonal regulation. The treatment of *P. brasiliensis* with female hormones such as oestrogens blocks the conidia- or mycelium-to-yeast transition [35–37], probably via a cytosolic steroid-binding protein [38], and the resistance of females to the disease appears attributable to this hormonal effect [39]. As oestradiol inhibits the morphological transition, altering the profiles of protein synthesis of mycelia and yeast, this suggests that the oestradiol–protein complex modulates protein expression in *P. brasiliensis*. An understanding of the molecular basis of this hormonal effect could provide novel approaches to block the development of the pathogenic form of the fungus.

Interestingly, in *C. albicans*, the ATP-binding cassette (ABC) transporter that confers resistance to azole drugs, Cdr1p [40], can also transport human steroid hormone and the expression of the transporter is upregulated by this hormone [41,42]. There is a correlation between the level of azole resistance in *C. albicans* and its ability to form hyphae in the presence of azole drugs [43]. Furthermore, steroid-binding proteins have been identified and related to the pathogenicity of *C. albicans* [44], and azole drugs can inhibit the binding of steroids to these binding proteins [45]. These observations indicate that steroid hormones can regulate morphological changes, pathogenicity and drug resistance. In our lab, we have identified related ABC-transporters in *P. brasiliensis* [C.H. Gray *et al*., (1999) The identification and characterization of *mdr* genes from *Paracoccidioides brasiliensis*, encoding putative drug efflux ABC transporters. Abstract, 2nd FEBS Advanced Lecture Course, ATP-Binding Cassette Transporters: From Multidrug Resistance to Genetic Disorders]. Accordingly, can we expect to see the azole-induced selection of strains of *P. brasiliensis* which, owing to upregulated expression of these pumps and the consequent conferred ability to extrude mammalian hormones, will lead to an increase in the incidence of paracoccidioidomycosis in females?

Adherence to host cells

An *in vitro* assay for the adhesion of *P. brasiliensis* to cultured mammalian epithelial cells has established a correlation between adherence and virulence, with virulent strains having a greater capacity to adhere [46,47]. In this model, yeast cells were shown to attach to the epithelial cells via a narrow tube (Fig. 1b) similar to a germ tube, which subsequently led to the yeast cells making close and extensive contact with the membrane of the host cell, inducing cytoskeletal changes before being taken up, probably by phagocytosis [47]. Could *P. brasiliensis* use the



TRENDS in Microbiology

Fig. 2. *Paracoccidioides brasiliensis* undergoes a temperature-induced morphological transition. The four panels show electron micrographs of (a) mycelium at 26°C, (b) mycelium grown at 36°C for 11 days, (c) mycelium grown at 36°C for 15 days and (d) yeast grown at 36°C for 21 days. Notice the transition from filamentous to budding growth in shifting the temperature from 26°C to 36°C. Scale bars = 10 μm. Reproduced, with permission, from [58].

tube to probe the epithelial cells for surface breaks, in analogy to the thigmotropic behaviour of *C. albicans* hyphae? The structure and purpose of the tube that makes the initial connection with the host cell has yet to be elucidated.

The adherence of *P. brasiliensis* to host epithelial cells is substantially reduced in the presence of anti-gp43 serum, indicating gp43 acts as an adhesin [46,47]. gp43 has been shown to bind to laminin of the basal membrane of the extracellular matrix (ECM) of mammalian tissues, and as laminin-coated yeast have an increased ability to invade and destroy infected tissues, gp43 on the surface of yeast cells has been proposed to act as a laminin receptor [48]. Furthermore, *P. brasiliensis* produces an exocellular serine-thiol protease that can cleave laminin and other components of the ECM [49]; however, gp43 is resistant to cleavage by this protease. The gene encoding gp43 has been cloned and sequenced, revealing similarity to exo-1,3-β-D-glucanases, but gp43 does not have any hydrolase activity, probably because the conserved glucanase active site amino acids Asn-Glu-Pro are Asn-Lys-Pro in gp43 [17]. The resemblance of gp43 to glucanases suggests a role in binding carbohydrates, possibly the carbohydrate moiety of laminin. In addition, other components of the ECM, such as fibronectin and collagens, have been shown to act as targets for the adhesion of *P. brasiliensis* [46].

Sialoglycoconjugates are exposed on the surface of *P. brasiliensis* cells [50] that have been implicated in the adhesion of fungal pathogens to host cells [51], possibly via the substantial negative charge that they impose upon the cell surface [52].

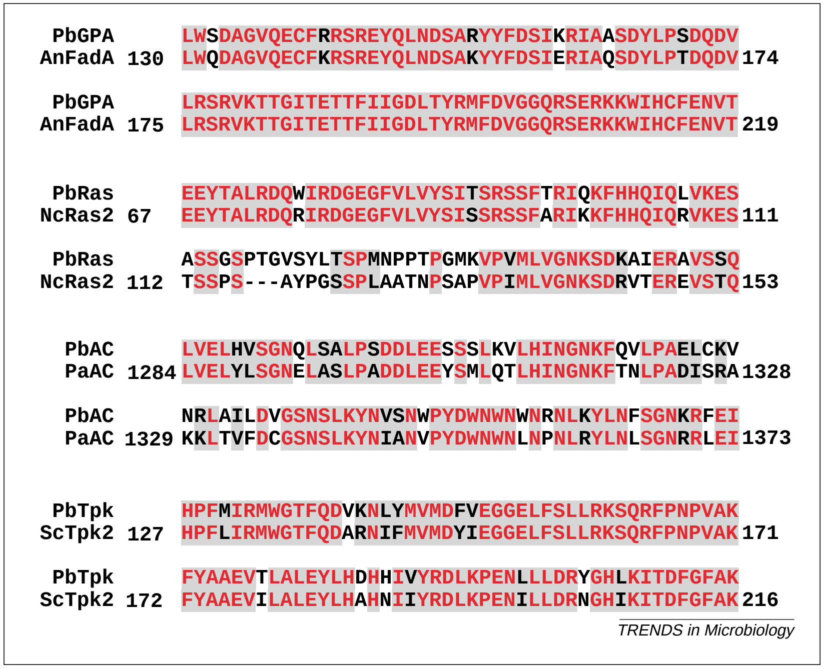
Morphological changes in *P. brasiliensis*

P. brasiliensis is a thermally dimorphic fungus that exists in a mycelial form at environmental temperatures (e.g. 26°C) and in a yeast form at the temperature of the mammalian host (37°C) [53]. The transition from mycelium to yeast can be induced *in vitro* by merely shifting the incubation temperature from 26°C to 37°C (Fig. 2). This behaviour contrasts with that of *C. albicans* in which infectivity is associated with the yeast-to-hyphal transformation and more complex stimuli than temperature alone, such as serum, are required to induce morphological switching, which does not proceed to completion, allowing hyphal, pseudohyphal and yeast cells to co-exist [54].

The cell signalling pathway

Little is known of the signalling pathways that control the morphological changes in *P. brasiliensis*, but the cAMP pathway is likely to be important because exogenous cAMP inhibits the yeast-to-mycelial transition and thus supports the pathogenic yeast form [55]. By contrast, exogenous cAMP supports the pathogenic filamentous forms of *C. albicans* and the yeast-to-hyphal transition is controlled by the cAMP pathway [56]. In our lab, we have identified the elements of a cAMP pathway in *P. brasiliensis*, and the genes encoding Ras, Gpa (G_{α}-protein subunit), protein kinase A and adenylate cyclase have been cloned (A.R. Walmsley *et al.*, unpublished) (Fig. 3). Further studies are required to elucidate the cell signalling pathways that control the morphological transition in *P. brasiliensis*. However, it will be of considerable interest to determine whether *P. brasiliensis* modulates related pathways to those driving hyphal and pseudohyphal formation in *C. albicans* and *Saccharomyces cerevisiae*, respectively, to drive the opposite morphological change and/or whether it uses homologues of *Candida* transcription factors (e.g. Tup1p and Nrg1p [57]) that repress hyphal formation to maintain the pathogenic yeast form.

Although *P. brasiliensis* does not appear to recognize the 26°C to 37°C transition as a true heat-shock, in which it downregulates the expression of most gene products in favour of specific heat-shock proteins (Hsp), the major Hsp, Hsp70, is differentially expressed in mycelia and yeast, with a progressive increase in Hsp70 expression during the 21-day period that it takes to convert from mycelium to yeast [58]. This appears to be controlled by the differential splicing of the introns from *hsp70*, which are only effectively removed by the yeast form. This implies that Hsp70 is required to stabilize the proteins necessary for the morphological transition. Interestingly, in *S. cerevisiae*, activation of the cAMP pathway, which can trigger the development of



TRENDS in Microbiology

Fig. 3. Identification of the components of a cAMP signal transduction pathway in *Paracoccidioides brasiliensis*. In each case, a representative 90 amino acid residues of the translated gene products, putatively encoding a G_{α}-protein (PbGPA), Ras (PbRas), adenylate cyclase (PbAC) and protein kinase A (PbTpk) proteins in *P. brasiliensis*, are aligned against the protein identified from a database search having the highest overall sequence similarity. These sequence alignments indicate the high degree of similarity of *P. brasiliensis* gene products to key elements of the cAMP signalling pathways of other fungi. In particular, the *P. brasiliensis* GPA, Ras, adenylate cyclase and Tpk proteins most closely resemble FadA from *Aspergillus nidulans* (AnFadA), which regulates sporulation, Ras2 from *Neurospora crassa* (NcRas2), which regulates hyphal and conidial development, adenylate cyclase from *Podospora anserina* (PaAC) and Tpk2p, the protein kinase A that regulates pseudohyphal growth in *Saccharomyces cerevisiae* (ScTpk2), respectively. Conserved residues are indicated in red and areas of similarity, allowing for conservative substitutions, are highlighted by grey boxes. The amino acid positions of the aligned sequences within the known fungal gene products are given.

filamentous pseudohyphae, causes an increase in the heat-shock sensitivity of the cells [55]. If the activation of the cAMP pathway in *P. brasiliensis* led to an increase in the heat-shock sensitivity, this would be detrimental to cells undergoing the mycelium-to-yeast transition, which occurs over several days. Is this difference in behaviour attributable to differences in the thermotolerance of *P. brasiliensis* and *S. cerevisiae* or has *P. brasiliensis* developed strategies to protect these gene products during the morphological transition? Recent studies have revealed that the activity of the cAMP pathway in *S. cerevisiae* depends upon the amount of Cdc25p, a guanine-exchange factor (GEF) that acts on Ras2p; the Hsp70 protein Ssa1p controls the cellular content of Cdc25p in *S. cerevisiae* [59]. An interesting hypothesis is that *P. brasiliensis* Hsp70 detects and stabilizes components of the cAMP signalling pathway to elicit the temperature-induced morphological transition.

Changes in the cell membrane and cell wall

The morphological change in *P. brasiliensis* is accompanied by extensive changes in the cell wall. Most notably, there is an increase in chitin content and a change in the glucan polymer linkage from β-1,3-glucan to α-1,3-glucan as the fungus adopts the yeast form [53]. Because neither chitin nor glucan is present in the mammalian host, these enzymes have great potential as drug targets. Furthermore, there has been great interest in the enzyme β-1,3-glucan synthase because it is the target of the echinocandin family of antifungal cyclic lipopeptides and a β-glucan synthase has been cloned from *P. brasiliensis* [60]. However, α-1,3-glucan synthase is of particular interest because the transition to the pathogenic yeast form is dependent upon the formation of α-glucan linkages within the cell wall. Indeed, in common with *Histoplasma capsulatum* and *Blastomyces dermatitidis*, mutant strains of *P. brasiliensis* have been isolated in which decreased amounts of cell wall α-1,3-glucan correlate with decreased virulence [61]. Only recently, a gene, termed *mok1*, encoding an α-glucan synthase that has little similarity to β-glucan synthases, was cloned from the fission yeast *Schizosaccharomyces pombe* [62]. Recent work in our lab indicates that *P. brasiliensis* possesses a gene that resembles *mok1* (A.R. Walmsley *et al.*, unpublished). Interestingly, β-glucan has been shown to trigger the inflammatory response of the cells of the lung [63], possibly accounting for the fact that avirulent strains of *P. brasiliensis* produce a greater inflammatory response than virulent strains because their cell walls contain less α-glucan and more β-glucan [64]. An interesting question is whether *P. brasiliensis* uses the switch to α-glucan to avoid stimulating the inflammatory response. *P. brasiliensis* has also been shown to possess several chitin synthase genes but no correlation has been found between the transcript levels and the chitin content of yeast and mycelia [65].

The lipid composition of the cell membrane also changes during the morphological transition. In particular, there are differences in the glycosphingolipids (GSL) of yeast and mycelial cell membranes [66,67]. The major neutral GSLs are β-glucopyranosylceramides (GlcCer), which differ in whether or not they have an unsaturated bond in the 18-carbon 2-hydroxy fatty acid side chain of the ceramide, and two major acidic glycosylinositol phosphorylceramides (GIPC), which differ in that one contains a β-galactofuranose. The different GlcCer and GIPC compounds are present at near equivalence in mycelial membranes but yeast membranes predominantly comprise the saturated GlyCer and the β-galactofuranose-containing GIPC. The β-galactofuranose-containing GIPC is reactive with antibodies from paracoccidioidomycosis patients [68] whereas the saturated GlyCer has been shown to be reactive with sera from patients suffering from cryptococcosis, and inhibits cell budding and growth of *Cryptococcus neoformans* [69]. Indeed, in *C. neoformans* the GlyCer is preferentially located at the sites of budding [69]. It is possible that these changes in the composition

Questions for future research

- Can an efficient transformation system be developed that will enable reverse genetics approaches to be applied to the discovery of gene function in *P. brasiliensis*?
- Does *P. brasiliensis* use the same cell signalling pathways to regulate the transition from the filamentous to the unicellular form that other fungi, such as *C. albicans*, use to drive hyphal development or has it developed novel signalling pathways? If related, will comparative genomics approaches with *C. albicans* allow us to identify how these signalling pathways are modified?
- What is the molecular basis of the advantage conferred on *P. brasiliensis* by changing the configuration of the glucan linkages during the morphological transition? Can we identify the genes involved in the cell signalling pathway that regulates this transition as targets for antimycotic drugs?
- What are the molecular mechanisms underlying the latency of *P. brasiliensis*?
- What is the molecular mechanism underlying the hormonal inhibition of the transition from infective conidia to parasitic yeast in *P. brasiliensis*? In particular, which protein binds steroid hormones and how does it transduce the signal to inhibit the morphological transition?

of the cell membrane are required to regulate the activity of the synthases (e.g. glucan and chitin synthase) and hydrolases (e.g. glucanases and chitinases) that modify the composition of the cell wall, because most of these proteins are integral to the membrane. Furthermore, there could be a more intricate connection between cell wall synthases and GSLs. First, by forming lipid microdomains, GSLs are involved in the sorting of glycosylphosphatidylinositol (GPI)-anchored proteins. Second, truncated GPI anchors are used to crosslink mannoproteins to glucans and chitin that are constituents of the cell wall. It is noteworthy that *P. brasiliensis* produces phospholipase C in abundance [70], which could be used to liberate GPI anchors, possibly from GIPC, for crosslinking to glucans and chitin.

Recent studies indicate that *P. brasiliensis* conidia and yeast cells produce melanins [71], an important finding because melanins have been implicated in the virulence of both plant and animal pathogens. In particular, the presence of melanins in *C. neoformans* protects the conidia against environmental stresses, by efficient scavenging of reactive oxygen species by the pigment, and against host defence mechanisms and antimicrobial therapies [72]. Whether melanin plays a similar role in *P. brasiliensis* awaits elucidation.

Acknowledgements
The Wellcome Trust supports the work in our laboratory on *P. brasiliensis*. Owing to restrictions on the number of references cited, it has not been possible to cite all the references related to *P. brasiliensis*. Accordingly, we have cited only those references that we felt were particularly pertinent, relying on review articles to cover earlier work, and more recent publications that are not discussed in these reviews

Concluding remarks

Our knowledge of *P. brasiliensis* is more limited than that of several other fungal pathogens, such as *C. albicans* and *C. neoformans*, which have been the subject of comparatively intense investigation. However, by inference from other fungal pathogens we can point to both comparable and distinct pathobiologies. For example, several putative virulence factors can be attributed to *P. brasiliensis*. Most notably, *P. brasiliensis* produces adhesins and extracellular proteases that allow it to attach to and degrade the ECM, and it undergoes a morphological transition from a saprobic mycelium to a pathogenic yeast, involving considerable changes in the composition of the cell membrane and cell wall, presumably to enhance its survival in the mammalian host. Indeed, *P. brasiliensis* uses a novel mechanism of differential intron splicing in mycelia and yeast to increase the level of expression of the major heat shock protein, which presumably regulates the activity of those gene products that are necessary for triggering and maintaining the yeast form. *P. brasiliensis* also produces melanin and phospholipase, which act as virulence factors in *C. neoformans*.

Does *P. brasiliensis* also share with *C. neoformans* the property of being a facultative intracellular pathogen [72]? *P. brasiliensis* can replicate in macrophages and epithelial cells and the host response to the pathogen is that of cell-mediated immunity and granuloma formation, responses that are usually associated with intracellular pathogens. Paracoccidioidomycosis is a persistent infection that often develops after a long period of latency, and many patients relapse following termination of treatment with antifungals. The inability to eradicate *P. brasiliensis* with drugs and its ability to disseminate from the lungs to other tissues and to lie dormant for several years might be the result of its intracellular survival. However, the mechanisms underlying these features of *P. brasiliensis* and the disease that it causes remain unknown.

Although our knowledge of *P. brasiliensis* is growing, clearly a major hurdle to the progression of these studies is the absence of transformation and gene-disruption systems that would allow the roles of genes of interest to be probed by reverse genetics. Work in our lab and others is progressing towards establishing molecular genetic techniques for *P. brasiliensis*. However, even in the absence of such molecular genetic approaches, comparative genomics between *P. brasiliensis* and other pathogenic fungi, such as *C. albicans*, and between different *P. brasiliensis* strains are likely to provide information on the virulence traits of this organism. Indeed, mutant strains of *P. brasiliensis* are available which are avirulent, virulent, only virulent after passage through the mammalian host, and with altered morphology (e.g. yeast at room temperature and yeasts with reduced α-glucan content). Comparisons of the patterns of gene expression in these mutant strains might allow us to identify virulence genes and genes that are important for switching to the pathogenic yeast form. We are just beginning to understand the pathogenicity of *P. brasiliensis* and much remains to be uncovered about this fascinating but deadly organism.

References

1 Brummer, E. *et al*. (1993) Paracoccidioidomycosis: an update. *Clin. Microbiol. Rev*. 6, 89–117
2 Restrepo, A. *et al*. (2001) The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? *Med. Mycol.* 39, 232–241
3 Cano, M.I.N. *et al*. (1998) Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. *J. Clin. Microbiol*. 36, 742–747
4 Montoya, A.E. *et al*. (1999) Electrophoretic karyotype of environmental isolates of *Paracoccidioides brasiliensis*. *Med. Mycol*. 37, 219–222
5 McEwan, J.G. *et al*. (1987) Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. *J. Med. Vet. Mycol*. 25, 165–175
6 Franco, M.M. (1994) Pathology. In *Paracoccidioidomycosis* (Franco, M. *et al*., eds), pp. 142–146, CRC Press
7 Mendes, R.P. *et al*. (1994) Treatment and control of cure. In *Paracoccidioidomycosis* (Franco, M. *et al*., eds), pp. 373–392, CRC Press
8 Restrepo, A. (2000) Morphological aspects of *Paracoccidioides brasiliensis* in lymph nodes: implications for the prolonged latency of paracoccidioidomycosis? *Med. Mycol*. 38, 317–322
9 Sherman, D.R. *et al*. (2001) Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding α-crystallin. *Proc. Natl. Acad. Sci. U. S. A.* 98, 7534–7539
10 Benard, G. *et al*. (1997) Immunosuppression in paracoccidioidomycosis: T cell hyporesponsiveness to two *Paracoccidioides brasiliensis* glycoproteins that elicit strong humoral responses. *J. Infect. Dis.* 175, 1263–1267
11 Benard, G. *et al*. (2000) Imbalance of IL-2, IFN-γ and IL-10 secretion in the immunosuppresion associated with human paracoccidioidomycosis. *Cytokine* 13, 248–252
12 Calich, V.L.G. *et al*. (1998) Immunity to *Paracoccidioides brasiliensis* infection. *Res. Immunol.* 149, 407–417
13 Franco, L. *et al*. (1998) Experimental pulmonary fibrosis induced by *Paracoccidioides brasiliensis* conidia: measurements of local host responses. *Am. J. Trop. Med. Hyg.* 58, 424–430
14 Forbes, J.R. and Gros, P. (2001) Divalent-metal transport by NRAMP proteins at the interface of host–pathogen interactions. *Trends Microbiol*. 9, 397–403
15 Moscardi-Bacchi, M. *et al.* (1994) Support of *Paracoccidioides brasiliensis* multiplication by human monocytes or macrophages: inhibition by activated phagocytes. *J. Med. Microbiol*. 40, 159–164
16 Kurita, N. *et al*. (2000) Effect of cytokines on antifungal activity of human polymorphonuclear leucocytes against yeast cells of *Paracoccidioides brasiliensis*. *Med. Mycol*. 38, 177–182
17 Cisalpino, P.S. *et al.* (1996) Cloning, characterization and epitope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis*. *J. Biol. Chem.* 271, 4553–4560
18 Bava, A.J. *et al*. (1991) Lymphocyte subpopulations and cytokine production in paracoccidioidomycosis patients. *Microbiol. Immunol*. 35, 167–174
19 Tapia, F.J. *et al*. (1991) Leukocyte immunophenotypes in branchoalveolar lavage fluid and peripheral blood of paracoccidioidomycosis, sarcoidosis and silicosis. *Histol. Histopathol*. 6, 395–402
20 Karhawi, A.S. *et al*. (2000) Production of IFN-γ is impaired in patients with paracoccidioidomycosis during active disease and is restored after clinical remission. *Med. Mycol.* 38, 225–229
21 Taborda, C.P. *et al*. (1998) Mapping the T-cell epitope in the major 43 kDa glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. *Infect. Immun*. 66, 786–793
22 Pinto, A.R. *et al*. (2000) DNA-based vaccination against murine paracoccidioidomycosis using *gp43* gene from *Paracoccidioides brasiliensis*. *Vaccine* 18, 3050–3058
23 Kashino, S.S. *et al*. (2000) Resistance to *Paracoccidioides brasiliensis* infection is linked to a preferential Th1 immune response, whereas susceptibility is associated with absence of IFN-γ production. *J. Interferon Cytokine Res.* 20, 89–97
24 Cano, L.E. *et al*. (1998) Protective role of γ interferon in experimental pulmonary paracoccidioidomycosis. *Infect. Immun*. 66, 800–806
25 Souto, J.T. *et al*. (2000) Interferon-γ and tumor necrosis factor-α determine resistance to *Paracoccidioides brasiliensis* infection in mice. *Am. J. Pathol.* 156, 1811–1820
26 Deepe, G.S. *et al*. (2000) Knockout mice as experimental models of virulence. *Med. Mycol.* 38, 87–98
27 Bocca, A.L. *et al*. (1998) Treatment of *Paracoccidioides brasiliensis*-infected mice with a nitric oxide inhibitor prevents the failure of cell-mediated immune response. *J. Immunol*. 161, 3056–3063
28 Gonzalez, A. *et al*. (2000) Nitric oxide participation in the fungicidal mechanism of γ interferon-activated murine macrophages against *Paracoccidioides brasiliensis* conidia. *Infect. Immun*. 68, 2546–2552
29 Cano, L.E. *et al*. (1994) Inhibitory effect of deferoxamine or macrophage activation on transformation of *Paracoccidioides brasiliensis* conidia by ingested macrophages: reversal by holotransferrin. *Infect. Immun*. 62, 1494–1496
30 Soares, A.M.V.C. *et al*. (2001) Modulatory effect of prostaglandins on human monocyte activation for killing of high- and low-virulence strains of *Paracoccidioides brasiliensis*. *Immunology* 102, 480–485
31 Souza, A.R. *et al*. (2000) Anti-idiotypic antibodies in patients with different clinical forms of paracoccidioidomycosis. *Clin. Diagn. Lab. Immunol*. 7, 175–181
32 Benard, G. and Duarte, A.J.S. (2000) Paracoccidioidomycosis: a model for evaluation of the effects of human immunodeficiency virus infection on the natural history of endemic tropical diseases. *Clin. Infect. Dis.* 31, 1032–1039
33 Cano, L.E. *et al*. (2000) Depletion of CD8$^+$ T cells *in vivo* impairs host defense of mice resistant and susceptible to pulmonary paracoccidioidomycosis. *Infect. Immun.* 68, 352–359
34 Restrepo, A. *et al.* (1997) Hormonal influences in the host-interplay with *Paracoccidioides brasiliensis*. In *Fungal Interactions* (Stevens, D.A. *et al*., eds), pp. 125–133, National Foundation for Infectious Diseases
35 Restrepo, A. *et al*. (1984) Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracocccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. *Infect. Immun.* 46, 346–353
36 Salazar, M.E. *et al*. (1988) Inhibition by estrogens of conidium-to-yeast conversion in the fungus *Paracoccidioides brasiliensis*. *Infect. Immun*. 56, 711–713
37 Clemons, K.V. *et al*. (1989) Influence of estradiol on protein expression and methionine utilization during morphogenesis of *Paracoccidioides brasiliensis*. *J. Gen. Microbiol.* 135, 1607–1617
38 Loose, D.S. *et al*. (1983) Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis*. *Proc. Natl. Acad. Sci. U. S. A.* 80, 7659–7663
39 Aristizabal, B.H. *et al*. (1998) Morphological transition of *Paracoccidioides brasiliensis* conidia to yeast cells: *in vivo* inhibition in females. *Infect. Immun.* 66, 5587–5591
40 Prasad, R. *et al*. (1995) Molecular cloning and characterization of a novel gene of *Candida albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr. Genet*. 27, 320–329
41 Krishnamurthy, S. *et al*. (1998) Characterisation of human steroid transport mediated by Cdr1p, a multidrug transporter of *Candida albicans*, belonging to the ATP binding cassette super family. *FEMS Microbiol. Lett*. 158, 69–74
42 Krishnamurthy, S. *et al*. (1998) Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiol. Lett*. 160, 191–197
43 Ha, K.C. and White, T.C. (1999) Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast *Candida albicans*. *Antimicrob. Agents Chemother*. 43, 763–768
44 Powell, B.L. *et al*. (1984) Identification of a 17-β-estradiol-binding protein in *Candida albicans* and *Candida (Torulopsis) glabrata*. *Exp. Mycol*. 8, 304–313
45 Stover, E.P. *et al.* (1983) Ketoconazole binds to the intracellular corticosteroid-binding protein in *Candida albicans*. *Biochem. Biophys. Res. Commun*. 117, 43–50
46 Mendes-Giannini, M.J.S. *et al*. (2000) Pathogenesis II. Fungal responses to host responses: interaction of host cells with fungi. *Med. Mycol*. 1, 113–123
47 Hanna, S.A. *et al*. (2000) Adherence and intracellular parasitism of *Paracoccidioides brasiliensis* in Vero cells. *Microbes Infect.* 2, 877–884
48 Vicentini, A.P. *et al*. (1994) Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect. Immun.* 62, 4–7
49 Puccia, R. *et al*. (1998) Exocellular proteolytic activity of *Paracoccidioides brasiliensis*: cleavage of components associated with the basement membrane. *Med. Mycol*. 36, 345–348
50 Soares, R.M.A. *et al*. (1998) Anionogenic groups and surface sialoglycoconjugate structures of yeast forms of the human pathogen *Paracoccidioides brasiliensis*. *Microbiology* 144, 309–314
51 Hamilton, A.J. *et al*. (1999) Recognition of fibronectin by *Penicillium marnefi* conidia via sialic acid-dependent process and its relationship to the interaction between conidia and laminin. *Infect. Immun*. 67, 5200–5205

52 Wasylnka, J.A. and Moore, M.M. (2000) Adhesion of *Aspergillus* species to extracellular matrix proteins: evidence for involvement of negative charged carbohydrates on the conidial surface. *Infect. Immun*. 68, 3377–3384

53 San Blas, G. and San Blas, F. (1993) Biochemistry of *P. brasiliensis* dimorphism. In *Paracoccidioidomycosis* (Franco, M. *et al.*, eds), pp. 49–66, CRC Press

54 Brown, A.J.P. and Gow, N.A.R. (1999) Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol*. 7, 333–338

55 Borges-Walmsley, M.I. and Walmsley, A.R. (2000) cAMP signalling in pathogenic fungi: control of dimorphic switching and pathogenicity. *Trends Microbiol.* 8, 133–141

56 Rocha, C.R.C. *et al*. (2001) Signaling through adenyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell* 12, 3631–3643

57 Braun, B.R. *et al*. (2001) *NRG1*, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *EMBO J*. 20, 4753–4761

58 da Silva, S.P. *et al*. (1999) The differential expression and splicing of an *hsp70* gene during the transition from the mycelial to the infective yeast form of the human pathogenic fungus *Paracoccidioides brasiliensis*. *Mol. Microbiol*. 31, 1039–1050

59 Geymont, M. *et al.* (1998) Ssa1p chaperone interacts with the guanine nucleotide exchange factor of ras Cdc25p and controls the cAMP pathway in *Saccharomyces cerevisiae*. *Mol. Microbiol*. 30, 855–864

60 Pereira, M. *et al.* (2000) Molecular cloning and characterization of a glucan synthase gene from the human pathogenic fungus *Paracoccidioides brasiliensis*. *Yeast* 165, 451–462

61 Hogan, L.H. and Klein, B.S. (1994) Altered expression of surface α-1,3-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence. *Infect. Immun*. 62, 3543–3546

62 Katayama, S. *et al*. (1999) Fission yeast α-glucan synthase Mok1 requires the actin cytoskeleton to localize the sites of growth and plays an essential role in cell morphogenesis downstream of protein kinase C function. *J. Cell Biol*. 144, 1173–1186

63 Vassallo, R. *et al*. (2000) Isolated *Pneumocystis carinii* cell wall glucan provokes lower respiratory tract inflammatory responses. *J. Immunol*. 164, 3755–3763

64 Silva, C.L. *et al*. (1994) Involvement of cell wall glucans in the genesis and persistence of the inflammatory reaction caused by the fungus *Paracoccidioides brasiliensis*. *Microbiology* 140, 1189–1194

65 Nino-Vega, G.A. *et al*. (2000) Differential expression of chitin synthase genes during temperature-induced dimorphic transitions in *Paracoccidioides brasiliensis*. *Med. Mycol*. 38, 31–39

66 Toledo, M.S. *et al*. (1998) Structure elucidation of sphingolipids from the mycopathogen *Paracoccidioides brasiliensis*: an immunodominant β-galacto-furanose residue is carried by a novel glycosylinositol phosphorylceramide antigen. *Biochemistry* 37, 8764–8775

67 Toledo, M.S. *et al*. (1999) Characterization of sphingolipids from mycopathogens: factors correlating with expression of 2-hydroxy fatty acyl (*E*)-Δ [3]-unsaturation in cerebrosides of *Paracoccidioides brasiliensis* and *Aspergillus fumigatus*. *Biochemistry* 38, 7294–7306

68 Toledo, M.S. *et al*. (1995) Glycolipids from *Paracoccidioides brasiliensis.* Isolation of a galactofuranose-containing glycolipid reactive with sera of patients with paracoccidioidomycosis. *J. Med. Vet. Mycol*. 33, 247–251

69 Rodrigues, M.L. *et al*. (2000) Human antibodies against purified glucosylceramide from *Cryptococcus neoformans* inhibits cell budding and fungal growth. *Infect. Immun*. 68, 7049–7060

70 Heise, N. *et al*. (1995) *Paracoccidioides brasiliensis* expresses both glycosylphosphatidylinositol-anchored proteins and a potent phospholipase C. *Exp. Mycol.* 19, 111–119

71 Gomez, B.L. *et al*. (2001) Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis in vitro* and during infection. *Infect. Immun.* 69, 5760–5767

72 Feldmesser, M. *et al*. (2001) Intracellular parasitism of macrophages by *Cryptococcus neoformans*. *Trends Microbiol*. 9, 273–278

The functions of Ca^{2+} in bacteria: a role for EF-hand proteins?

Jan Michiels, Chuanwu Xi, Jan Verhaert and Jos Vanderleyden

In bacteria, Ca^{2+} is implicated in a wide variety of cellular processes, including the cell cycle and cell division. Dedicated influx and efflux systems tightly control the low cytoplasmic Ca^{2+} levels in prokaryotes. Additionally, the growing number of proteins containing various Ca^{2+}-binding motifs supports the importance of Ca^{2+}, which controls various protein functions by affecting protein stability, enzymatic activity or signal transduction. The existence of calmodulin-like proteins (containing EF-hand motifs) in bacteria is a long-standing hypothesis. Analysis of the prokaryotic protein sequences available in the databases has revealed the presence of several calmodulin-like proteins containing two or more authentic EF-hand motifs, suggesting that calmodulin-like proteins could be involved in Ca^{2+} regulation in bacteria.

EF-hand proteins are ubiquitous in eukaryotes and fulfil important regulatory or buffering roles. Calmodulin, a prototypical EF-hand protein, is a small (15–22 kDa), acidic (pI 3.9–4.3) Ca^{2+}-binding protein that can interact with >25 distinct target proteins, thereby regulating the activity of many vital enzymes, including kinases, phosphatases, nitric oxide synthases, phosphodiesterases and ion channels [1]. Calmodulin is composed of four EF-hands organized in two pairs linked by a flexible central tether (Fig. 1a). The EF-hand motif was first discovered in the crystal structure of parvalbumin [2] and comprises two nearly perpendicular α-helices (named after helices E and F in parvalbumin) separated by a 12-residue loop [3]. In general, EF-hand Ca^{2+}-binding proteins are recognized by homology in their Ca^{2+}-binding loops. Residues 1, 3, 5, 7, 9 and 12 of the loop provide the ligands for complexing Ca^{2+} ions and form the basis of a pentagonal bipyramidal coordination geometry (Fig. 2). Residues 1, 3 and 5 provide monodendate ligands, and residue 12 a bidendate ligand, via side-chain carboxylates. Residue 7 coordinates Ca^{2+} via a main-chain oxygen and residue 9 coordinates Ca^{2+} indirectly via a water molecule. The identity of the amino acids in the loop has a profound effect on the Ca^{2+}-binding characteristics of the protein [4]. Pairs rather than individual EF-hands constitute the

http://tim.trends.com 0966-842X/01/$ – see front matter © 2001 Elsevier Science Ltd. All rights reserved. PII: S0966-842X(01)02284-3