Calcium Binding by the Essential Virulence Factor BAD-1 of Blastomyces dermatitidis*

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BAD-1 (Blastomyces adhesin 1), a 120-kDa protein of Blastomyces dermatitidis, functions as an adhesin, immune modulator, and essential virulence factor. Structurally, BAD-1 is composed of a short N-terminal region, a core of 30 tandem repeats critical for virulence, and a C-terminal epidermal growth factor domain that binds the protein to yeast cell surface chitin. Each of the 30 acidic residue-rich tandem repeats contains a sequence that resembles the calcium-binding loop of the EF-hand domain found in many calcium-binding proteins. Here, we investigated the binding of calcium by BAD-1 and its biological significance. Yeast washed with double distilled H₂O released surface-bound BAD-1, but EGTA washes were an order of magnitude more efficient, suggesting an interaction between BAD-1 and calcium. Immobilized BAD-1 was stained with ruthenium red dye, an indicator of calcium-binding proteins. In equilibrium dialysis, BAD-1 bound ⁴⁵Ca²⁺ with an affinity of 0.41 × 10⁻⁵ M and a capacity of 27 calcium/mol. Mass spectrometry confirmed this capacity. Elevated [Ca²⁺] diminished BAD-1 solubility. Upon deletion of its C-terminal epidermal growth factor-like domain, BAD-1 resisted aggregation by elevated [Ca²⁺] but retained its affinity and capacity for calcium. Removing 20 copies of the tandem repeat, however, sharply reduced the capacity of BAD-1 for calcium. Growth of the bad-1 null yeast was inhibited by 5 mM EGTA, and re-expression of BAD-1 in trans or the addition of exogenous purified BAD-1 restored growth. Thus, BAD-1 is a high capacity calcium-binding protein. This property contributes to the structure and function of BAD-1, as well as to B. dermatitidis acquisition of calcium from the environment.

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‡The abbreviations used are: EGF, epidermal growth factor; ddH₂O, double distilled H₂O; TSP, thrombospondin; ICP, inductively coupled plasma.
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Stock cultures were maintained in the yeast form on slants of Middlebrook 7H10 agar with oleic acid–albumin complex (Sigma) at 39 °C. Liquid cultures were grown in Erlenmeyer flasks containing *Histoplasma* macrophage medium (20) at 37 °C in a New Brunswick floor model gyratory incubator shaker at 200 rpm. Calcium limitation medium (3M-trace Ca2+) was based on the recipe for 3M minimal medium but contained only trace calcium (2.5 μM/liter) (7).

Preparation of BAD-1 Protein—Full-length BAD-1 protein can be isolated with a high degree of purity by simply washing *B. dermatitidis* yeast with buffer and then extracting the surface BAD-1 protein with three sequential 1-h-long water washes, but proteins were engineered to display a six-histidine tag in the transgenic strains to allow an additional purification step on a nickel affinity agarose (nickel-nitrilotriacetic acid) column (Qiagen) as previously described (6). Nickel-nitrilotriacetic acid purification is applied directly to the C-terminally truncated BAD-1 protein released in culture supernatants because it does not adhere to the yeast surface and must be purified from culture medium (7). Washing and protein elution were performed according to the manufacturer’s specifications.

All of the proteins employed in this work were verified to yield one predominant band upon silver stain of a PAGE gel separation (Fig. 1B). Faint bands flanking the predominant band were found to bind anti-BAD-1 monoclonal antibody DD5-CB4 (21, 22) in Western analysis, indicating that they are degradation products or multimers of the protein.

Experimental Procedures

Strains and Media—*B. dermatitidis* strains used in this study included ATCC wild-type strain 26199, *bad-1* knock-out strain 55 derived from 26199 (5), and several derivatives of strain 55 in which full-length BAD-1 or truncated derivatives were re-expressed in trans including: BAD1–6H, which expresses a six-histidine-tagged BAD-1 protein (intact or full-length BAD-1) (7); ΔC-term-6H, which expresses the tagged BAD-1 protein minus the C-terminal EGF-like domain (ΔC-term) (7); and ΔRepeat20–6H, which expresses the tagged BAD-1 protein minus 20 (of 30) tandem repeats (ΔRepeat20) (Fig. 1A).

Construction of yeast strains expressing intact BAD1–6H and ΔC-term–6H have been described (7). The ΔRepeat20–6H derivative was produced by digesting the plasmid pBAD1–6H (7) with BamH1 and religating, thus deleting the coding sequence for 20 of the 30 tandem repeats while maintaining the reading frame. The sequence of the resultant plasmid was confirmed and used to transform strain 55. Stable transformants were screened to select a strain with protein production comparable with wild-type strain 26199.

![FIGURE 1. Production of tagged and deleted BAD1 proteins. A, domains of native BAD1 (top) and the intact BAD1, ΔC-term, and ΔRepeat20 constructs (below). A six-histidine tag was placed at the C terminus of each construct using PCR. A 286-bp sequence was deleted from the C-terminal coding region in the ΔC-term construct, resulting in the loss of the EGF-like domain (95 amino acids) from the expressed protein. A 1501-bp sequence was deleted from the tandem repeat coding region in the ΔRepeat20 construct, resulting in the loss of 500 amino acids from the expressed protein. B, silver stain analysis of BAD-1 purity by PAGE. BAD1 transgenic proteins purified by nickel-nitrilotriacetic acid column appear as a single predominant band. Protein migration is commensurate with expected molecular weights (ΔM, denoted on right). Faint bands either below or above the primary bands were stained with anti-BAD-1 monoclonal antibody in Western analysis, indicating that they are degradation products or multimers of the protein.](image-url)
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CaCl₂ at concentrations of 0.5, 10, 20, 40, and 60 mM in a volume of 1 ml. The samples were incubated for 30 min at 24 °C with agitation, after which the tubes were spun down for 10 min at 14,000 rpm in a microcentrifuge. Precipitation was quantified by measuring a decrease in soluble phase BAD-1 using a Beckman DU-64 Spectrophotometer at A₃₈₀.

Equilibrium Dialysis—Purified BAD-1 protein (1 mg/ml) was treated with 20 mM EDTA, placed into dialysis tubing (Pierce), and then dialyzed against 10 mM Tris-HCl, pH 7.4, for 24 h with three 1-liter exchanges at 4 °C (to minimize residual calcium). Dialysis buffer was treated with Chelex-100 (Sigma) prior to use to remove trace calcium. Equilibrium dialysis analysis consisted of dialyzing the calcium-free protein in a Slide-A-Lyzer MINI dialysis unit (Pierce) against Tris-HCl buffer, pH 7.4, containing varied concentrations of calcium (5, 10, 25, 50, 100, 200, 300, and 400 μM). Dialysis buffer was spiked with 1.25 μCi of ⁴⁰CaCl₂, and dialysis proceeded for 18 h at room temperature with orbital rotation. 0.5% Tween 20 was added to both the protein sample and calcium buffers to minimize complications caused by calcium-induced protein self-aggregation (25). Radioactivity in both the protein sample and the dialysate was assessed via scintillation counter (Beckman LS 6000TA). The protein concentrations of the samples were measured post-dialysis by BCA assay (Pierce). The molar quantity of calcium bound to the protein sample, and thereafter the molar ratio of calcium to protein, was calculated as previously described (26). To minimize calcium contamination, only plasticware (polypropylene and polystyrene) was utilized for these assays. Atomic emission spectrometry (Galbraith Laboratories, Knoxville, TN) ensured that buffers used in this protocol were calcium-free (<0.5 μM).

Inductively Coupled Plasma Mass Spectrometry and Optical Emission Spectrometry Analysis—The Soil and Plant Analysis Lab at the University of Wisconsin–Madison analyzed the calcium content of samples of purified BAD-1 protein using inductively coupled plasma (ICP) mass spectrometry (VG PlasmaQuad PQ2 Turbo Plus ICP-MS) and ICP optical emission spectroscopy (TJA IRIS Advantage ICP-OES). Standard Soil and Plant Analysis Lab procedures were followed in the analysis of total minerals and heavy metals.

Prepared samples included fresh, intact BAD-1 that was: 1) untreated, 2) treated with 50 mM EGTA, pH 8, for 1 h at 24 °C with agitation, or 3) exposed to 3 mM CaCl₂ for 1 h at 24 °C with agitation. Preparations of ΔC-term and ΔRepeat20 protein were treated similarly and analyzed in parallel. Unbound calcium and EGTA were removed to trace levels (<8 parts/billion) via a desalting step involving multiple rounds of ultrafiltration in a Microcon® YM-50 unit (Millipore, Bedford, MA).

Growth of B. dermatitidis under Calcium Limiting Conditions—Strains of 26199 (wild type), strain 55 (bad-1 knock-out), and BAD1–6H (BAD-1 reconstituted knock-out) yeast were washed with 0.1 M EGTA two times for 5 min each. Flasks of minimal fungal medium, 3M-trace Ca²⁺ (containing trace calcium at 2.5 μM/liter) were inoculated with 1 × 10⁵ yeast of each strain at the outset of the experiment. In parallel, the cultures were treated as follows: 1) 5 mM EGTA was added to one set of cultures, 2) 5 mM EGTA + 5 mM CaCl₂ was added to a second set, and 3) nothing was added to the control set. The cultures were incubated at 37 °C and 200 rpm, and growth was determined over the course of the experiment by removing aliquots to measure A₆₀₀ and count yeast on a hemacytometer.

CD, Spectrofluorophotometry, and UV Absorbance Spectroscopy—UV absorption spectra over the 200–350-nm range were collected with a Beckman DU-64 spectrophotometer at 25 °C, in quartz cuvettes with a 1-cm light path. The spectra were recorded for 50 μg/ml BAD-1 in 10 mM Tris, pH 7.4, and for 50 μg/ml BAD-1 in 10 mM Tris, pH 7.4, plus 300 mM calcium chloride. Base-line spectra for buffer and buffer plus calcium, respectively, were subtracted from experimental spectra (27).

Fluorescence spectroscopy data were collected using a Shimadzu RF-5301PC spectrofluorophotometer. The excitation wavelength was 295 nm, and emission data were collected between 300 and 400 nm. All of the measurements were performed in 10 mM Tris buffer, pH 7.4, 0.15 M NaCl, at 25 °C using quartz cuvettes with a 3 mm light path. BAD-1 was used at a final concentration of 50 μg/ml. Calcium chloride of 100–300 mM was added. The spectra were collected for protein with and without calcium (28).

Near- and far-UV CD spectra were recorded with an Aviv 62A DS circular dichroism spectrometer at the Biophysics Instrumentation Facility at the University of Wisconsin-Madison Biochemistry Department. The temperature was set to 25 °C, and the work was done under nitrogen gas using quartz cuvettes with a 0.1-cm light path. The CD spectra were recorded for 50 μg/ml BAD-1 in 10 mM Tris, pH 7.4, and for 50 μg/ml BAD-1 in 10 mM Tris, pH 7.4, plus 300 mM calcium chloride. Base-line spectra for buffer and buffer plus calcium, respectively, were then subtracted from the experimental spectra.

Trypsin Digestion—BAD-1 (1 mg/ml) was incubated with calcium (final concentration, 1.25 mM) or not (EGTA; final concentration, 1.25 mM) and digested with trypsin (protein:trypsin ratio of 1:15). As a control, bovine serum albumin (1 mg/ml) was similarly treated with trypsin in the presence and absence of calcium. The reactions were performed in HEPES buffer, pH 7.0, at 50 °C for varying intervals of 1 min to 2 h (29, 30). The reactions were stopped by mixing samples with 2× PAGE gel loading buffer and heating for 3 min at 95 °C. The samples were kept on ice until they were run on 12% SDS-PAGE gels. The gels were stained with Coomasie Blue, and duplicate gels were immunoblotted. For immunoblotting, the proteins were transferred overnight at 30 V from PAGE gels onto polyvinylidene difluoride membranes. Non-specific proteins were blocked for 1 h with phosphate-buffered saline containing 5% milk. The blots were incubated with primary chicken antibody (1:500) raised against the EGF-like domain of the BAD-1 C terminus (Aves Labs, Inc. Tigard, OR). Secondary antibody of anti-chicken IgY (Promega, Madison, WI) was used at a dilution of 1:2000.

RESULTS

BAD-1 Sequence Shows an EF-hand-like Motif—Each of the 24-amino acid tandem repeats present in the core of BAD-1 displays homology to the calcium-binding loop of the EF-hand consensus sequence (Fig. 2) (8, 9). However, these sequences do not adhere precisely to the canonical EF-hand sequence: 1) a histidine instead of a glycine is present at position 6 and 2) a lysine instead of an isoleucine or some other
aliphatic residue occupies position 8. The tandem repeats also lack the α-helical motifs that normally flank each calcium-binding loop in an EF-hand (8, 9). The acidic loops found in BAD-1 do not precisely duplicate any other known type of calcium-binding domain, to our knowledge, but the absence of α-helical flanking sequences is not unprecedented (e.g. -TSP) (17). Our prior finding that BAD-1 binding to yeast cell surfaces depends on the concentration of calcium (10), taken together with the fact that BAD-1 consists of 30 EF-hand-like motifs strung together, raised the possibility that BAD-1 could be a calcium-binding protein.

BAD-1 Release from the Yeast Surface—In prior work, we showed that BAD-1 could be removed from the yeast by washing the cells in ddH2O (10). Inclusion of 5 mM calcium during these extractions retarded the release of BAD-1, whereas the inclusion of 5 mM magnesium did not, suggesting that this release hinges on the depletion of calcium ions. Here, we extended these studies. During three consecutive 10-min washes in ddH2O, BAD-1 was released into the soluble phase (Fig. 3A). The addition of a calcium chelator, 10 mM EGTA, pH 8, for an equivalent length of time released >10-fold more BAD-1 protein. The addition of 11 mM CaCl2 to this EGTA extraction completely inhibited BAD-1 release (data not shown). These results show that BAD-1 attachment to the yeast cell surface and subsequent release are dependent upon calcium, but they are insufficient alone to demonstrate calcium binding by BAD-1.

Ruthenium Red Staining of BAD-1—Staining with ruthenium red dye is frequently employed in the detection and indication of calcium-binding proteins following electrophoresis or dot blotting (24). Compared with an RNase A control, which has no calcium binding activity, intact BAD-1 protein immobilized on a nitrocellulose filter was stained by ruthenium red (Fig. 3B). The observation that ruthenium red binds preferentially to BAD-1 suggests a calcium binding capacity for BAD-1. Ruthenium red does not bind exclusively to calcium-binding proteins, however, and can also stain polyanionic substrates, so this observation alone is not conclusive.

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Equilibrium Dialysis of BAD-1 Calcium Binding—To definitively evaluate the binding capacity and affinity of BAD-1 for calcium, we performed equilibrium dialysis (Fig. 4 and TABLE ONE). Intact BAD-1 protein led to the enrichment of 45Ca2+ in the dialysis chamber over a wide range of calcium concentrations tested. At 10 μM, calcium occupied 15% of the theoretical BAD-1 binding sites, and this rose to 67% at 100 μM. Nonlinear regression analysis indicated a dissociation constant ($K_d$) of 41.3 ± 6.8 μM (n = 9 independent experiments). ΔC-term exhibited a maximal binding of 25 calcium ions/monomer and a $K_d$ of 62.2 ± 16.9 μM (n = 6). ΔRepeat20 exhibited a maximal binding of 11 calcium ions/monomer and a $K_d$ of 39.7 ± 8.6 μM (n = 4).
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The calcium to BAD-1 ratios for intact BAD-1 and derivatives were measured by mass spectrometry and equilibrium dialysis and compared to a non-calcium binding control (RNase A). ΔC-term lacks the C-terminal EGF region, and the ΔRepeat20 has 20 of the 30 tandem repeats deleted. Mass spectrometry showed that the purified proteins retained negligible calcium after EGTA extraction, and except for the RNase A control, each monomer bound 1–3 calcium ions in the absence of calcium saturation. Calcium saturation was achieved by incubating proteins with 3 mM CaCl₂ for 1 h followed by repeated ultrafiltration to remove unbound calcium. ICP mass spectrometry was done as described under “Experimental Procedures” and confirmed by optical emission spectroscopy (not shown). The \( K_d \) values were calculated by nonlinear regression analysis.

| BAD-1 and derivatives | \( \text{Ca}^{2+} \) ions/monomer | \( K_d \) \( \mu M \) |
|-----------------------|---------------------------------|-----------------|
| Intact BAD-1          | 27                              | 27              |
| ΔC-term               | 23                              | 25              |
| ΔRepeat20             | 10                              | 11              |
| RNase A Control       | <0.25                           | ND              |

Certain calcium binding proteins that include a hydrophobic domain in their sequence (e.g. calmodulin, thrombospondin, and recoverin) undergo a conformational switch when exposed to calcium. They display this domain for binding to receptors (when present). In the absence of their receptor, some of them may aggregate because of hydrophobic interactions involving such domains, especially at higher concentrations of protein. We explored the contribution of the EGF-like domain of BAD-1 to binding and aggregation by deleting this domain from ΔC-term. In contrast to intact BAD-1, the ΔC-term protein did not visibly precipitate upon the addition of calcium, and 85–89% of the protein was detectable in the supernatant at even the highest concentrations of calcium (Fig. 5). Thus, the C-terminal EGF-like domain fosters self-aggregation of BAD-1 upon calcium saturation.

We considered the alternate possibility that the results above might be due to the loss of calcium binding by ΔC-term BAD-1, because EGF domains have been reported to bind calcium in other proteins (31). To evaluate calcium binding in the absence of the C-terminal EGF region, we performed equilibrium dialysis on ΔC-term BAD-1 and the intact protein in parallel (Fig. 4B and TABLE ONE). ΔC-term still bound considerable calcium. At 10 \( \mu M \), calcium occupied 2% of the theoretical binding sites in ΔC-term, and at 100 \( \mu M \) this value rose to 63%. Nonlinear regression analysis indicated a \( K_d \) of 62.2 ± 16.9 \( \mu M \), and maximal binding was calculated to be 25 calcium ions/molecule of ΔC-term. Thus, capacity for calcium binding was reduced by only two calcium ions in the ΔC-term protein despite its markedly different physical response to elevated \( [\text{Ca}^{2+}] \) (versus intact BAD-1).

Although removal of the C-terminal EGF-like domain reduced calcium-binding from 27 to 25 ions in equilibrium dialysis, most of the capacity for calcium binding remained, implying that the tandem repeats contribute most significantly to the binding of calcium. We explored this hypothesis by investigating ΔRepeat20 truncated BAD-1. Binding of calcium by this derivative was sharply reduced (Fig. 4C and TABLE ONE). At 10 \( \mu M \), calcium occupied 11% of the theoretical binding sites in ΔRepeat20, and at 100 \( \mu M \) this value rose to 66%. Nonlinear regression analysis indicated a \( K_d \) of 39.7 ± 8.6 \( \mu M \), and maximal binding was calculated to be 11 calcium ions/molecule of ΔRepeat20. Hence, most of the capacity for binding calcium lies within the tandem repeat domain of BAD-1.

ICP-Mass Spectrometric Analysis and ICP-Optical Emission Spectroscopic Analysis of BAD-1—Both ICP mass spectrometry and ICP optical emission spectroscopy analyses were performed to ascertain the molar ratio of calcium ions/intact BAD-1, as well as ΔC-term and ΔRepeat20 and confirm the results obtained by equilibrium dialysis. Calcium content of the proteins was assayed under various conditions: 1) without added calcium; 2) saturated with calcium, and 3) after the protein was extracted with EGTA. Calcium-saturated, intact BAD-1, ΔC-term and ΔRepeat20 were found to bind 27, 23, and 10 calcium ions/mol of protein, respectively (TABLE ONE). In a low calcium environment, intact BAD-1, ΔC-term, and ΔRepeat20 retained about two calcium ions/mol of protein. The data for calcium-saturated proteins is remarkably similar to that generated by equilibrium dialysis. This confirms the high capacity of BAD-1 for binding calcium, which is reduced only slightly by removal of the C-terminal EGF domain and markedly by removal of 20 tandem repeats.

Investigation of BAD-1 Conformation Change by Circular Dichroism, Spectrofluorometry, and Tryptic Digestion—We sought evidence of a conformational change to explain the interaction of BAD-1 with itself or its receptor on exposure to calcium. The addition of calcium to calcium-stripped samples of BAD-1 (27, 28) produced changes in absorbance and fluorescence that were too small to be considered significant (data not shown). We saw no significant change in the UV-CD spectrum of this protein when it was taken from a calcium-depleted state to calcium saturation.

Digestion of BAD-1 with trypsin, however, was sharply influenced by the presence of calcium. BAD-1 was reduced to peptides quickly, within 1 min, especially in the presence of calcium. In the absence of calcium (1 mM EGTA), BAD-1 was more resistant to digestion (Fig. 6A) at all time points analyzed from 1 to 120 min. Western blots of the digests probed with antibody to the C-terminal EGF domain showed that several of the partially digested fragments retain the EGF domain and are relatively resistant to digestion in the absence of calcium, especially the ~40-kDa fragment (Fig. 6B, note arrows in the lanes with calcium-free digests). Digests of bovine serum albumin as a control were unaffected by the presence of calcium or EGTA (data not shown). BAD-1 incubated with calcium or EGTA in the absence of trypsin showed no evidence of degradation or autodigestion (data not shown).

![Figure 5. Role of the C-terminal EGF region in calcium precipitation of BAD-1.](image)
BAD-1 Induces Calcium to Associate with Yeast Cells—We explored whether the calcium binding activity of BAD-1 enhances association of calcium with the B. dermatitidis yeast cell. Incubation of heat-killed yeast of wild-type strain 26199 with $^{45}\text{CaCl}_2$ led to the subsequent association of radioactive calcium with the yeast (Fig. 7A). In contrast, bad-1 knock-out strain 55 incubated under the same conditions showed significantly reduced uptake. The addition of purified, intact BAD-1 to the knock-out strain, which we previously showed binds to and coats the surface of the yeast (7), restored $^{45}\text{CaCl}_2$ uptake in this strain, whereas the presence of EGTA blocked the uptake of calcium mediated by exogenous BAD-1.

To explore the biological consequence of calcium binding in BAD-1, we performed functional assays. We first sought to discover whether the presence of BAD-1 influenced the ability of B. dermatitidis to withstand calcium limitation. Wild-type strain 26199 and knock-out strain 55 grew comparably well in 3M minimal medium containing 2.5 mM calcium (3M-trace Ca$^{2+}$ medium). When these strains were cultured in 3M-trace Ca$^{2+}$ medium + 5 mM EGTA (effectively a 2000-fold excess of EGTA with respect to calcium), however, we observed a significant difference in their ability to grow (Fig. 7B). The wild-type strain grew well in this medium (doubling time, $\sim$24 h). The bad-1 knock-out strain showed nearly no growth for several weeks but began to grow again after this delay. Re-expression of BAD-1 in trans in the knock-out strain restored its ability to grow in this calcium-poor environment, and the BAD-1 complemented strain showed growth kinetics similar to that of the wild-type strain. Furthermore, the loss of expression of the transgene in this strain (upon removal of selection in vitro) was associated with retarded growth as in the knock-out strain (data not shown).

To further test the effect of BAD-1 complementation, purified BAD-1 was "spiked" into calcium-limited cultures of bad-1 knock-out strain 55 at 0.0, 0.1, and 1.0 $\mu$g/ml (the latter corresponding to the concentration of BAD-1 secreted by 26199 in minimal medium) (Fig. 7C). A concentration of 1 $\mu$g/ml BAD-1 reversed the fungistatic effects of the EGTA significantly, whereas lower concentrations or no BAD-1 did not. Thus, under the calcium-limiting growth conditions studied, B. dermatitidis strains producing endogenous BAD-1 or provided exogenous BAD-1 had a significant advantage in obtaining essential calcium, even in the presence of an excess of EGTA.
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DISCUSSION

Many of the known calcium-binding proteins belong to the same evolutionary family and share a type of calcium-binding domain known as the EF-hand. This domain consists of a 12-residue loop flanked on both sides by short α-helical motifs. In an EF-hand loop, the calcium ion is coordinated by a pentagonal bi-pyramidal arrangement of oxygens contributed by side chains and main chain carboxyls. The residues involved in calcium binding are typically in positions 1, 3, 5, 7, 9, and 12 (denoted by X, Y, Z, −Y, −X, and −Z; see Fig. 2). Although we have referred to the tandem repeats as resembling the highly familiar EF-hand consensus sequence, it is probably more accurate to draw comparisons with the type 3 repeats present in TSP. The repeats in TSP include the calcium-binding loop portion of the EF-hand helix-loop-helix motif but lack the E and F helices that normally frame and stabilize that loop (17). TSP closes and constrains its highly conserved calcium-binding loops with a cysteine linkage instead, and whereas the cysteine bonding pattern of BAD-1 is unknown, it is notable that each one of its tandem repeats is bounded by a pair of cysteine residues (Fig. 2). It is also interesting that the calculated $K_d$ of BAD-1 for calcium ($41 \mu M$) is in the same range as that of TSP ($52 \mu M$) (18).

Perhaps one of the more interesting conclusions of our research is the discovery that ~80% of the BAD-1 molecule is almost certainly involved in the binding of calcium. Although the crystal structure of BAD-1 has not yet been solved, the EF-hand like sequences present within the tandem repeat region suggest certain necessities of structure. It seems more than coincidence that the number of nearly identical tandem repeats making up the central portion of BAD-1 is very nearly equal to the measured calcium binding capacity of the protein. It seems likely that each of the repeats enforces one calcium ion, with each of the coordinating residues contributing one oxygen molecule to the liganding interaction (or two in the case of the glutamate in the −Z position, which typically donates an additional oxygen in canonical EF-hand loops) (8) (Fig. 8).

If, in view of the similarities between BAD-1 and TSP, we further posit that the two cysteines in each BAD-1 repeat form a sulfhydryl bond to stabilize the calcium-binding loop, then there is no stretch of the tandem repeat region longer than five residues that is not involved directly or indirectly with the calcium binding modality. Because most of BAD-1 is composed of tandem repeats, and the C-terminal region is dispensable for virulence (7), it would appear that the segments of BAD-1 responsible for pathogenesis are heavily devoted to the function of binding calcium.

Calcium-binding proteins have been categorized as being either calcium sensors or calcium buffers by da Silva and Reinach (32). Proteins in the former category tend to bind two, four, or occasionally as many as eight calcium ions, whereas those in the latter category typically bind more (33). TSP is somewhat exceptional among calcium sensors, binding 12 calcium ions/monomer (18). TSP mediates many functions: adhesion and migration of cells, platelet aggregation, regulation of proliferation, as well as a capacity to bind to glycoproteins of the extracellular matrix. TSP possesses a variety of purpose-specific elements to mediate these interactions, but each of the functions is similarly dependent upon the binding of calcium (18). BAD-1 is likewise a multifunctional protein, mediating adherence to complement type 3 receptors, immune deviation, and yeast cell wall scaffolding, in addition to the binding of calcium described here. Calcium “sensing”/binding may serve to trigger these BAD-1 functions. At the same time, the binding of calcium itself might be a key function of BAD-1 much as siderophores bind and deliver iron to microbes, given that BAD-1 does promote fungal growth under calcium limiting conditions. In this regard, and in view of its exceptional number of calcium-binding motifs, BAD-1 appears to have more in common with calcium buffering proteins than calcium sensors.

BAD-1 behaves like certain other calcium-binding proteins in that it aggregates in the presence of calcium. Recoverin (34), ALG-2 (11), α-hemolysin (35), and calsequestrin (36) also precipitate in the presence of elevated [Ca$^{2+}$]. Structural modeling based on x-ray crystallography (37), NMR (33), and fluorescence spectra (12) indicate that upon binding calcium these proteins undergo a conformational shift in which hydrophobic residues become exposed on the surfaces of the proteins. These hydrophobic surfaces mediate interactions with a target receptor. In the absence of the appropriate receptor, the interaction of these surfaces with one another can lead to aggregation (12).

We found here that ΔC-term, harboring a C-terminal truncation of BAD-1, does not undergo self-aggregation in response to elevated [Ca$^{2+}$], and neither does it anchor itself on yeast cell surfaces (7). These findings highlight the importance of the C terminus in these functions. They imply that this domain would be integral to any calcium-induced conformational variation of BAD-1 that parallels the conformational switching seen in...
other well characterized calcium-binding proteins. We had hypothesized that the hydrophobic C-terminal domain might associate itself with hydrophobic residues present in the interior of the calcium-free form of BAD-1 and fold outward from this niche as BAD-1 takes up calcium. A realignment of the tryptophans in the tandem repeats, in response to the coordination of calcium, might facilitate this association.

Most of the 151 tryptophans in BAD-1 are located in proximity to the calcium-binding loops of the repeat. In computer modeling of the calcium-bound configuration, these tryptophans array themselves on the outer rims of the calcium-binding loops. The lack of a significant shift in CD spectra in response to calcium does not eliminate the possibility of realignment of tryptophan residues with subtle changes in conformation. It is possible that the cysteine bonds that flank each putative calcium-binding loop restrain any gross changes in the secondary structure of the protein.

Nevertheless, tryptic digests of BAD-1 in the presence and absence of calcium showed different peptide maps and C-terminal EGF fragment sizes. These findings are consistent with the premise that BAD-1 changes its conformation upon binding of calcium and by virtue of unfolding and displaying its C-terminal EGF exposes sites for tryptic digestion and faster degradation of this domain. The pathogenic role of the ~40-kDa EGF fragment stabilized by EGTA is not known at this time.

The mechanism by which calcium binding of BAD-1 impacts virulence of B. dermatitidis during infection remains speculative. There may be parallels between BAD-1 and the calcium-binding proteins secreted by microbial residents of the phagocyte endosome. Toxoplasma gondii, Mycobacterium tuberculosis, and Histoplasma capsulatum (one of the closest phylogenetic relatives of B. dermatitidis) all release calcium-binding proteins into the endosome during intracellular parasitism (13, 16, 38). T. gondii produces an intraphagosomal membrane network that coats T. gondii cells in the presence of 1 mM Ca2+ and is released from cell surfaces in a calcium-poor environment, much like BAD-1 (16). M. tuberculosis secretes lipoparabionannamin, a toxin that interferes with the maturation of phagosomes by blocking a novel Ca2+-calmodulin-Pi3K hVPS34 cascade. H. capsulatum secretes calcium-binding protein, a protein that allows H. capsulatum yeast to survive in a calcium-limited environment (conferring resistance to the fungistatic effects of EGTA). Calcium-binding protein is believed to scavenge calcium ions in the macrophage endosome, promoting survival of the yeast (13). Thus, the ability of BAD-1 to promote fungal survival under calcium limiting conditions could exert its influence during several steps of the host-parasite interaction.

We show here that BAD-1 confers a siderophore function by providing calcium to the fungus under calcium-limiting conditions (Fig. 7). BAD-1 also is known to act by deviating host cytokine responses, especially tumor necrosis factor-α and transforming growth factor-β (2–4). Soluble BAD-1 penetrates host cells (3) where it could bind calcium, alter cellular calcium fluxes, and perturb calcium-dependent signaling of pro-inflammatory cytokines (39). BAD-1 also accesses endosomes (3), where the binding of calcium could retard acidification, as described by Gerasimenko et al. (19), and hinder phagosome defenses.

In summary, we describe here a novel, high capacity calcium binding function for BAD-1, an essential fungal virulence factor. The large number of binding sites for calcium in BAD-1 and the necessity of the protein for fungal growth under calcium-limiting conditions underscore the biological significance of this newly described function. The influence of calcium binding on BAD-1 pathogenic function offer intriguing new areas of study in elucidating fungal virulence.