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Ubiquitin-Like Epitopes Associated with *Candida albicans* Cell Surface Receptors

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We have recently reported the cloning of a *Candida albicans* polyubiquitin gene and the presence of ubiquitin in the cell wall of this fungus. The polyubiquitin cDNA clone was isolated because of its reactivity with antibodies generated against the candidal 37-kDa laminin-binding protein. In the present study, we have further investigated the relationship between ubiquitin and cell wall components displaying receptor-like activities, including the 37-kDa laminin receptor, the 58-kDa fibrinogen-binding mannoprotein, and the candidal C3d receptor. Two-dimensional electrophoretic analysis and immunoblot experiments with antibodies against ubiquitin and the individually purified receptor-like molecules confirmed that these cell surface components are ubiquitinated. In an enzyme-linked immunosorbent assay, polyclonal antisera to each receptor reacted with ubiquitin, thus demonstrating that the purified receptor preparations used as immunogens contained ubiquitin-like epitopes. It is proposed that ubiquitin may play a role in modulating the activity of these receptors and in the interaction of *C. albicans* cells with host structures.

Ubiquitin is a small (approximately 8,500 Da) polypeptide first isolated from bovine thymus (13). Primary structural analysis of various ubiquitins and their encoding genes has revealed remarkable evolutionary conservation among species (11). In higher eukaryotes, ubiquitin plays important roles in protein modification, protein degradation, gene transcription, organization of chromatin structure, and stress resistance (11, 12). It is also associated with some cell surface proteins, including different receptor molecules (7, 15, 19, 21, 22, 25).

In a recent report we described the isolation of clones coding for polyubiquitin by screening of a *Candida albicans* cDNA library with antibodies generated against the candidal 37-kDa laminin-binding protein (23). Also, we reported the presence of several proteins in the cell wall of *C. albicans* that bear ubiquitin-like epitopes (23). The cell wall of *C. albicans* is a multilayer structure external to the plasma membrane that, as the outermost part of the cell, mediates the interaction between the fungus and its environment (6). A number of components on the cell surface of this fungus display receptor-like activities mediating binding of the fungal cells to host cells and tissues (for a review of this topic, see reference 2). In the present study, we have further investigated the relationship between ubiquitin and the 37-kDa laminin-binding protein of *C. albicans*. Observations have been extended to other cell wall components and more specifically to the 58-kDa fibrinogen-binding mannoprotein (5) and the C3d receptor (22, 26).

C. albicans 3153A was propagated as blastoconidia at 24°C in a medium first described by Lee and colleagues (14). β-Mercaptoethanol (βME) was used to solubilize cell wall components from intact cells as previously reported by our group (3, 4). The solubilized material was dialyzed, concentrated by freeze-drying, and resuspended in deionized water (βME extract). The total sugar content of the extract was determined by the method of Dubois et al. (10) using mannose as a standard.

Aliquots of the βME-extracted material were subjected to two-dimensional polyacrylamide gel electrophoresis (PAGE) by the method of O'Farrell (20) using a minigel system (Bio-Rad Laboratories, Hercules, Calif.). Polypeptides were separated in the first dimension under nonreducing conditions in nonequilibrium pH gradient electrophoresis (NEPHGE) tube gels with a pH range from 6 to 9. After equilibration, tube gels were placed on top of sodium dodecyl sulfate–12.5% PAGE gels, and the second dimension was run. The materials present in the gels were either stained with silver or transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) with a semidry electroblotter. The membrane was then incubated in the presence of a preparation of pooled rabbit antisera against *C. albicans* cell wall components (17), used at a final 1:1,000 dilution in Tris-HCl (10 mM; pH 7.4) containing 0.05% Tween 20 and 1% bovine serum albumin (TBSTB). Bound antibody was detected with a peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad; 1:2,000 dilution in TBSTB). In agreement with previous reports (3, 4, 8), silver staining of the separated materials (Fig. 1A) showed a complex protein pattern associated with the cell wall of *C. albicans*. However, the use of a two-dimensional system enabled an increased resolution of the different components present in the extract and detection of different moieties within the same molecular range, adding a new insight into the complexity of the *C. albicans* cell wall protein composition. Immunoblot analysis of the same materials with pooled antisera generated against *C. albicans* cell wall components (17) confirmed a complex antigenic composition of the cell wall extract (Fig. 1B). Immunoblots in which incubation with a first antibody was omitted or in which a rabbit preimmune serum was used as the first antibody were negative.

Only a few of these components were recognized by monoclonal (Chemicon, Temecula, Calif.) and rabbit polyclonal (Sigma Chemical Co., St. Louis, Mo.) antibodies against ubiquitin (used at final dilutions of 1:500 and 1:100 in TBSTB, respectively). Components exhibiting apparent molecular masses of 37, 40, and 58 kDa were reactive with the antiubiquitin antibodies. The immunoblot obtained with polyclonal antibody (PAb) against ubiquitin is shown in Fig. 2A. The presence of a 37-kDa moiety that contains ubiquitin-like epitopes in this extract could explain the fact that clones encoding ubiquitin were

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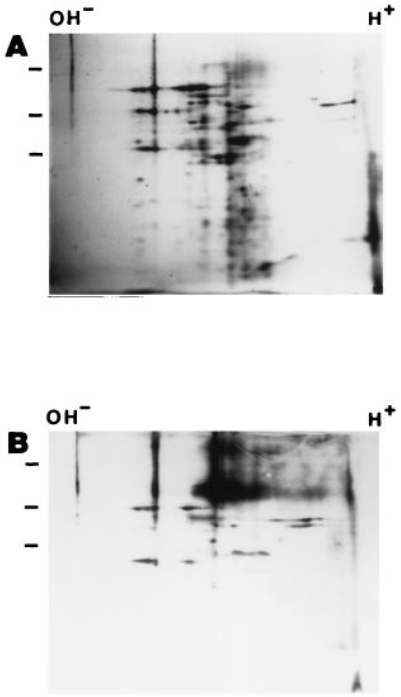

FIG. 1. Two-dimensional analysis of βME extracts from yeast cells of *C. albicans* 3153A. (A) Total protein composition of the extract as determined by silver staining. (B) Immunoblot analysis with a preparation of pooled polyclonal antisera against cell wall components. The amount of material loaded in each gel was 300 μg, expressed as total carbohydrate content. Molecular masses of standard proteins run in the second dimension are indicated with bars (from top to bottom, 72, 42, and 31 kDa) on the left of each panel.

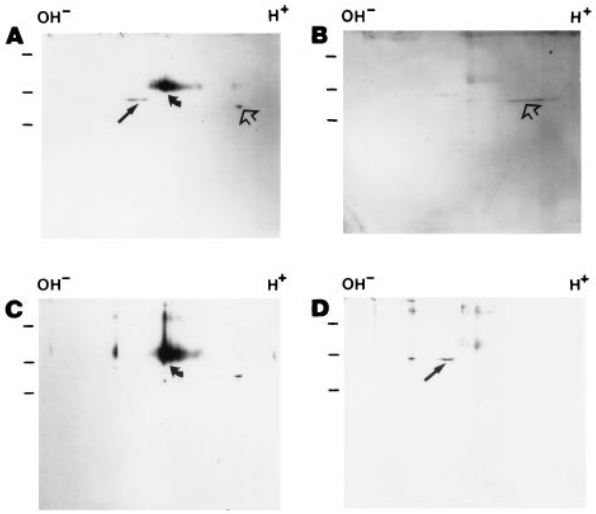

FIG. 2. Immunoblot analysis of components present in the βME extract from intact yeast cells. Components were separated by two-dimensional gel electrophoresis and detected with a polyclonal antiserum against ubiquitin (A), polyclonal antisera against the 37-kDa laminin-binding protein of *C. albicans* (B), the 58-kDa fibrinogen-binding mannoprotein (C), and the candidal C3d receptor (D). Open arrows in panels A and B indicate a 37-kDa component, curved arrows in panels A and C point to a 58-kDa component, and solid arrows in panels A and D indicate a 40-kDa species as detected by the antiubiquitin antiserum and each of the individual antisera against the different receptor-like components, respectively. The amount of sample applied in each gel was 300 μg, expressed as total sugar content. Molecular masses of standard proteins run in the second dimension are indicated with bars on the left of each panel (from top to bottom, 72, 42, and 31 kDa).

isolated when a cDNA library of *C. albicans* was screened with a rabbit PAb against the candidal 37-kDa laminin-binding protein. Colocalization of signals for both antiubiquitin (Fig. 2A) and anti-p37 (PAb anti-p37, used at a final dilution of 1:250 in TBSTB [Fig. 2B]) antibodies in the immunoblots with two-dimensionally separated components (Fig. 2A and B, open arrows) confirmed that this surface receptor was ubiquitinated. We considered the possibility that the other two ubiquitinated components, exhibiting apparent molecular masses of 40 and 58 kDa, were also previously identified components associated with binding activities. Two possibilities were the 58-kDa fibrinogen-binding mannoprotein (mp58) and one of the polypeptides recognized by the antiserum generated against the candidal receptor for C3d (PAb anti-CR2) (5, 18). Again, colocalization of reactive polypeptides was detected with both the antiubiquitin antibody (Fig. 2A) and the rabbit antisera against the individual purified receptors, i.e., PAb anti-mp58 (1:250 in TBSTB) (Fig. 2C) and PAb anti-CR2 (1:1,000 in TBSTB) (Fig. 2D). These observations indicated that these two receptor-like molecules on the surface of *C. albicans* contain ubiquitin-like domains.

Since these receptors are ubiquitinated, polyclonal antisera prepared against the purified receptors could also contain antibodies to ubiquitin epitopes. To confirm this possibility, the reactivities of the different polyclonal antisera with purified yeast ubiquitin (Sigma) were examined in an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed by standard procedures as previously described (18). As shown in Fig. 3, all three polyclonal antisera were able to recognize ubiquitin, thus demonstrating the presence of ubiquitin-like epitopes in the purified receptor preparations used as immunogens. Antiserum to the candidal C3d receptor showed the greatest reactivity against ubiquitin, whereas low levels of reactivity were detected with anti-mp58 antiserum. Each of these receptor molecules was originally purified by a different technique (5, 22, 23), thus making it very unlikely that ubiquitin nonspecifically copurified with each receptor. Also, the presence of antiubiquitin antibodies in the different antiserum preparations would predict cross-reactivity with other molecules bearing ubiquitin epitopes. In accord with this prediction, some cross-reactivity was observed in the immunoblots (Fig. 2B to D).

In recent years, the finding of ubiquitinated cell surface molecules has opened new avenues for the investigation of the location and functions of ubiquitin. At the cell surface, the

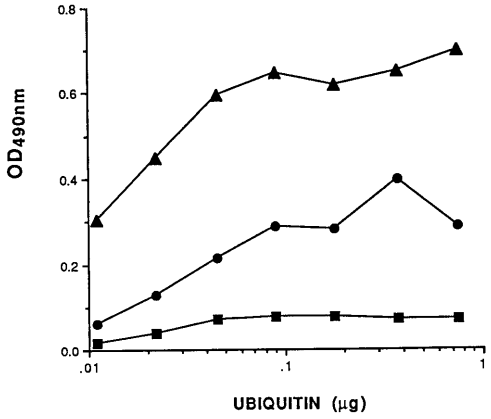

FIG. 3. Presence of antiubiquitin antibodies in the polyclonal antisera against the purified candidal receptors as detected by ELISA. A series of wells in a microtiter plate were coated with decreasing amounts of yeast ubiquitin and probed with PAb anti-CR2 (1:1,000 dilution) (▲), PAb anti-p37 (1:250) (●), and PAb anti-mp58 (1:250) (■). The second antibody was a peroxidase-conjugated goat anti-rabbit serum. Data represent binding to immobilized ubiquitin. OD_{490nm}, optical density at 490 nm.

close association between ubiquitin and different receptors suggests a role for ubiquitination in adhesion. These receptors include the mouse lymph node-specific homing receptor, the platelet-derived growth factor receptor, the growth hormone receptor, the high-affinity immunoglobulin E receptor, and the T-cell antigen receptor (7, 15, 19, 21, 24). Cell surface receptors are increasingly recognized as multisubunit complexes in which some of the subunits contain the domains used for interaction with ligands whereas others are used in signaling events. In this context, ubiquitin may play important roles in both ligand-binding and signaling activities. For *C. albicans*, the large repertoire of receptor-like molecules (2) may confer on the fungal cells an enormous potential to interact with different structures in the host, and receptor-mediated signaling events have been recently reported (1). We have recently described the presence of ubiquitinated cell surface proteins, although the exact nature of these proteins was unknown (23). The isolation of clones coding for ubiquitin by screening of a *C. albicans* cDNA library with antibodies generated against the candidal 37-kDa laminin-binding protein (16, 23) led us to further investigate a possible relationship between these two molecules. High-resolution two-dimensional gel electrophoresis followed by immunoblotting was used to demonstrate that the same moiety reacted with antibodies against p37 and ubiquitin. Observations were extended to two other well-characterized receptor-like molecules of *C. albicans*, i.e., the 58-kDa fibrinogen receptor mannoprotein (5) and the C3d receptor (22, 26). Ubiquitin-like epitopes were found to be associated with both components. Proteins are ubiquitinated by action of ubiquitin-conjugating enzymes. A family of genes coding for such enzymes has been described for fungi. One member of this family (*UBC4*) has been cloned in *C. albicans* (9).

Candidal cell surface receptors are often glycosylated, and different glycosylation patterns have been reported for the candidal C3d receptor (26). In other systems both carbohydrates and ubiquitin moieties are attached to a central core epitope (24). The combinatorial possibilities of ubiquitin conjugation to a number of cell surface proteins and ubiquitination of different sites of an individual protein, together with differences in glycosylation, may confer a great degree of variability on the different receptors in the interactions with their ligands. Overall, these results support the hypothesis that ubiquitin is associated with different receptor-like components on the surface of *C. albicans*. Ubiquitination may play a role in modulating the activity of these receptors and thus the ability of *C. albicans* cells to adhere to host cells and tissues.

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