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Development of Molecular Immunolabeling : scFv Antibodies Designed with Metal-Binding Domains

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Abstract

To study the molecular structure and function of gene products in situ, we developed a molecular immunolabeling technology. Starting with cDNA from hybridomas producing monoclonal antibodies against biotin, catalase, and superoxide dismutase, we bioengineered recombinant single-chain variable fragment antibodies (scFv) and their derivatives containing metal-binding domains (scFv:MBD). As tested with surface plasmon resonance and enzyme-linked immunosorbent assay, affinity binding constants of the scFv (5.21 × 106 M−1) and scFv:MBD (4.17 × 106 M−1) were close to those of Fab proteolytic fragments (9.78 × 106 M−1) derived from the parental IgG antibodies. After saturation of MBD with nickel or cobalt, scFv:MBD was imaged with electron spectroscopic imaging at each element's specific energy loss, thus generating the element's map. Immunolabeling with scFv:MBD resulted in a significant improvement of the labeling fidelity over that obtained with Fab or IgG derivatives, as it produced a much heavier specific labeling and label-free background. As determined with radioimmunoassay, labeling effectiveness with scFv:MBD was nearly the same as with scFv, but much higher than with scFv conjugated to colloidal gold, Nanogold, or horseradish peroxidase. This technology opens possibilities for simultaneous imaging of multiple molecules labeled with scFv:MBD at the molecular resolution within the same sample with electron spectroscopic imaging. Moreover, the same scFv:MBD can also be imaged with fluorescence resonance energy transfer and lifetime imaging as well as positron emission tomography and magnetic resonance imaging. Therefore, this technology may serve as an integrative factor in life science endeavors.

INTRODUCTION

The cell membrane of Saccharomyces cerevisiae is a primary site of heavy metal toxicity by Cd2+ and Cu2+, with resultant loss of mobile cellular solutes, such as K+ (1, 10, 13, 19). Silver, in addition to loss of K+, has been reported to increase efflux of accumulated phosphate, mannitol, succinate, glutamine, and proline (17, 18). Mercury and silver both inhibit yeast respiration. A specific target for mercury has not been defined, but ATP content of the cell is rapidly depleted (5). Silver is reported to bind with phosphate, resulting in collapse of the proton motive force (17). Toxic metal ions, including Cu2+, Co2+, Ni2+, Cd2+, Mn2+, and Hg2+, also inhibit plasma membrane ATPase by means of various binding interactions (15). Silver and mercury have relatively high affinities for reduced thiol groups, but which tain electrochemical gradients or membrane potential (2). Therefore, it is possible to use membrane damage as an indicator of heavy metal toxicity.

Propidium iodide (PI), which fluorescently stains nucleic acids in damaged or dead cells, has been widely used to indicate cell membrane integrity (8), whereas oxonols, which are lipophilic anionic dyes, accumulate in cells with reduced membrane potential (9). As long as the free dye concentration is below the saturation point for the binding sites available in the cell, the intracellular dye accumulation is membrane potential dependent (14). The relationship between changes in oxonol fluorescence and membrane potential is linear (9). The compound bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Ox) has the highest degree of voltage sensitivity among oxonols (3).

metric procedure with PI and Ox. The effects of Ag(I) and Hg(II) on both yeasts differed regarding loss of membrane integrity, membrane potential, and cell recoverability.

MATERIALS AND METHODS

Culture maintenance and growth

Cultures of C. albicans strain GSU-30 and C. maltosa strain R-42 were obtained from the lyophilized culture collection at Georgia State University. Stock cultures were maintained on Bacto Sabouraud dextrose agar (SAB; Difco Laboratories, Detroit, Mich.) slants and transferred every 3 to 4 weeks. Working cultures were grown on the Bacto yeast nitrogen base (Difco Laboratories) supplemented with 0.5% glucose (pH 5.5) (DYNB) with agitation at room temperature (22°C) for 18 h.

Chemicals

AgNO3 and HgCl2 (ACS reagent grade; Sigma Chemical Co., St. Louis, Mo.) were dissolved in deionized distilled water (ddH2O) to make 100 mM stock solutions. Working solutions of 0.005 to 0.20 mM concentrations were comprised of serial dilutions of the stock solutions in morpholineethanesulfonic acid (MES) buffer (J. T. Baker, Phillipsburg, N.J.). MES, which exhibits negligible metal-binding properties (11), was used for metal exposures at pH 5.5. MES at pH 6.8 was used for Ox staining (MES at this pH provided the greatest peak separation between heat-killed cells and live cells [data not shown]).

Exposure of Cells to Heavy Metals

Cells grown on DYNB for 18 h at 22°C were harvested in a centrifuge, washed twice with ddH2O, and suspended in MES buffer (pH 5.5) to an optical density at 600 nm of 0.6 in a 1.0-cm light path (equivalent to 2.33 × 106 cells of C. maltosa ml−1 or 1.20 × 107 cells of C. albicans ml−1). Various concentrations of Ag(I) or Hg(II) in 20 μl were added to 0.98 ml of cell suspension in 1.7-ml microcentrifuge tubes. The tubes were centrifuged and then incubated in static culture at room temperature for 1 h. These suspensions were then centrifuged at 10,000 × g for 2 min and the pellet was resuspended immediately in the staining buffers.

Fluorescent probe staining procedure

PI and Ox (Molecular Probes Inc., Eugene, Oreg.) were used separately for examination of membrane integrity and membrane potential, respectively. The protocols used for Ox and PI staining were similar to those described by Deere et al. (7). The stock solution of Ox contained 1.0 mM Ox in dimethyl sulfoxide (J. T. Baker), whereas the stock solution of PI contained 1.0 mg of PI ml−1 in phosphate-buffered saline (PBS). For Ox staining, yeast cells were suspended in MES (pH 6.8) to give approximately 106 to 108 cells ml−1. Stock dye solution (5.0 μl) was added to 1.0 ml of yeast suspensions. Samples were incubated for 30 min at room temperature in the dark before flow cytometric analysis. For PI staining, 20 μl of stock solution was added to 0.98 ml of yeast suspensions in PBS. The incubation time was always from 5 to 8 min before analysis.

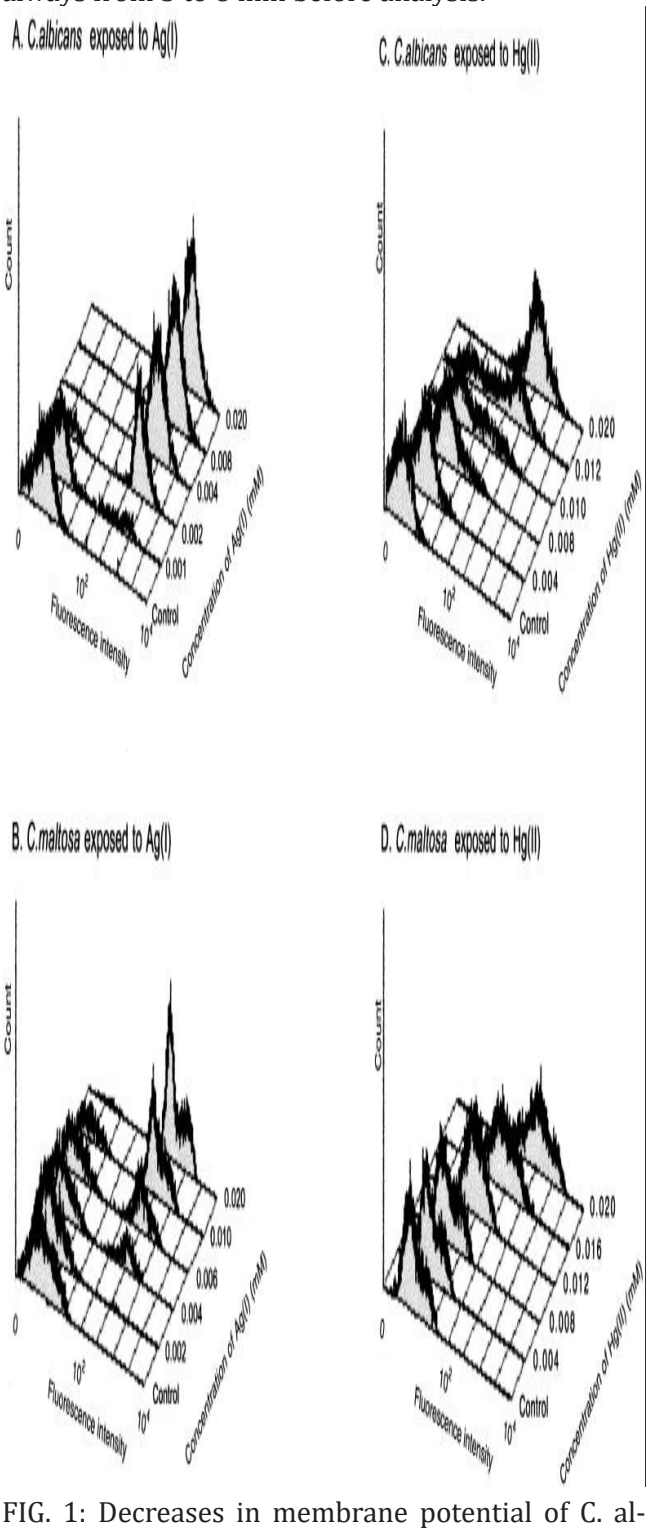

FIG. 1: Decreases in membrane potential of C. albicans (1.17 × 107 cells ml−1 [A], 1.98 × 107 cells ml−1 [C]) and C. maltosa (1.15 × 107 cells ml−1 [B], 7.40 × 106 cells ml−1 [D]) with exposure to increasing concentrations of Ag(I) and Hg(II) in MES buffer.

The depolarization rates for cells of C. albicans and C. maltosa exposed to Ag(I) and Hg(II) for different time periods are given in Fig. Fig.2.2. The cells

of both species showed similar responses. At a concentration of 0.02 mM Ag(I), most cells of C. maltosa lost their membrane potential within 2 min, whereas 0.02 mM Hg(II) had a negligible effect on membrane potential even at 15 min. The percentage of depolarized cells increased gradually after 15 min. At concentrations below 0.02 mM, the percentage of depolarized cells in Ag(I) reached a plateau (84%) within 15 min, but the percentage of depolarized cells in Hg(II) continued to increase with time (Fig. (Fig.2B).2B).

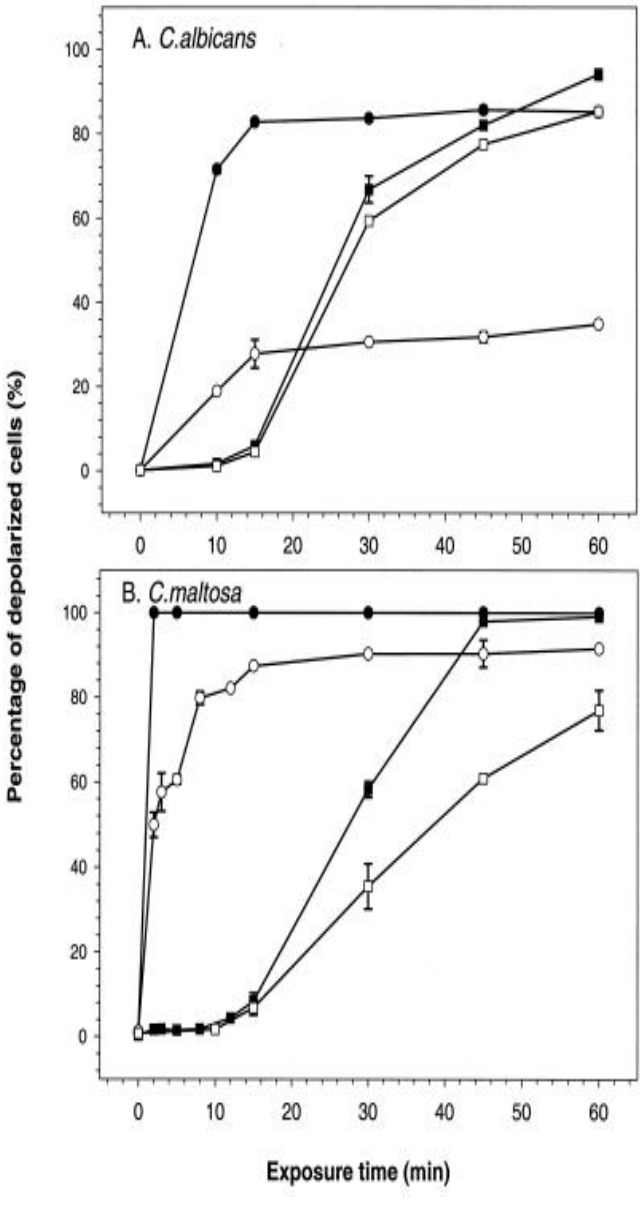

FIG. 2: (A) Depolarization with time of cells of C. albicans in 0.002 mM Ag(I) (●, 3.65 × 106 cells ml−1; ○, 7.38 × 106 cells ml−1) and 0.016 mM Hg(II) (▪, 3.65 × 106 cells ml−1; □, 7.38 × 106 cells ml−1). (B) Depolarization of C. maltosa (2.33 × 106 cells ml−1) in Ag(I) (●, 0.020 mM; ○, 0.004 mM) and Hg(II) (▪, 0.020 mM; □, 0.010 mM). Each data point represents the average obtained from duplicate independent assays.

asterisks, MMP-9; open circles, MMP-13. Inhibition is expressed as percentage activity, when 0–11μM calprotectin is present. Each point represents the mean of duplicates.

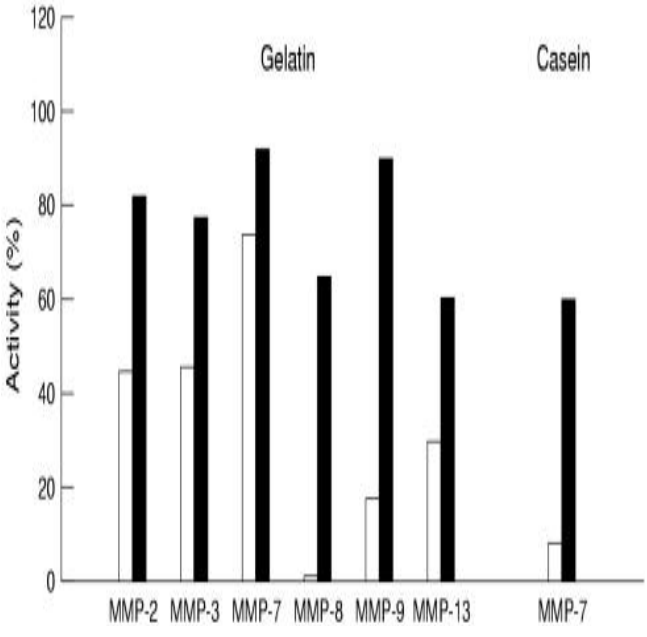

Figure 3: Relative activities of metalloproteinases in the gelatinolytic and caseinolytic microwell assays, when incubated with 11μM calprotectin and 1μM (open bars) or 100μM (closed bars) zinc. The figures are expressed as percentage activity compared with activity without calprotectin.

Discussion

Our results show that modifications of the method described by Rucklidge and Milne allow the quantitative determination of MMP activities. This method avoids the use of radioactive isotopes and different substrates can be used. Furthermore, the assay system is simple and sensitive, allowing detection of 3 ng/ml or less. However, this method is more time consuming than a recently described method using biotinylated gelatin.14 Another aspect is that some substrates, such as collagen, may be altered and less available for enzymatic degradation as a result of the coating process or exposure to paraformaldehyde. For instance, collagen type 1 (from calf skin, Fluka, Buchs, Switzerland) was almost completely converted into gelatin, which was shown by the fact that it was rapidly degraded by trypsin (data not shown).

MMPs are activators of a broad range of cytokines, including interleukin 1, tumour necrosis factor α, Fas ligand, and transforming growth factor β,15–19 and thereby play important roles in regulating processes such as acute and chronic inflammation, tumour cell invasion, apoptosis, and macrophage chemotaxis. Calprotectin may affect various pathophysiological processes by competing with MMPs for zinc. Our study revealed that calprot

ectin inhibits the activity of all the enzymes tested, and that this inhibition was overcome by the addition of zinc. A higher concentration of calprotectin was necessary to inhibit some metalloproteinases than others, regardless of the substrate. In the gelatinolytic assay, MMP-3, MMP-8, and MMP-13 needed a 200–700 times molar excess of calprotectin to give a 50% inhibition. By comparison, up to a 18 000 times molar excess was necessary to give a similar inhibition of MMP-2 and MMP-9.

These results suggest that MMPs have different affinities for zinc, and that calprotectin has an even lower affinity, because a large excess was necessary for inhibition.

Structurally, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 have one catalytic domain containing the zinc binding site. In addition, MMP-2 and MMP-9 have one zinc binding site closer to the C-terminal, suggesting a higher capacity for binding of zinc. MMP-7, the smallest of the proteins, also has one catalytic domain.1 Nonetheless, a much higher concentration of calprotectin was needed to inhibit this enzyme than MMP-3, MMP-8, or MMP-13, which suggests that MMP-7 has a higher affinity constant for zinc.

The metalloproteinases are totally dependent on zinc for their enzymatic activities,1 and our results support the hypothesis that some biological effects of calprotectin are linked to its sequestration of zinc. Sohnle et al showed that calprotectin inhibits microbial activity via a zinc deprivation mechanism,8,20 and it has also been shown that the apoptosis inducing activities of calprotectin were inhibited by the addition of micromolar concentrations of zinc.21 The concentrations of calprotectin needed to inhibit the MMPs in vitro may be biologically relevant. During bacterial infections, up to 120 ng/µl has been found in plasma.4 The release of calprotectin from neutrophils in human peripheral blood may give a concentration of about 20 ng/µl plasma, based on a content of 5 pg calprotectin/cell,22 and 4 × 109 neutrophils/litre blood. Local accumulation of granulocytes corresponding to five times the normal may provide 5µM calprotectin, which would lower the activity of most of the enzymes by 50% or more, if their concentrations in vivo were similar to those used in vitro. The enormous numbers of leucocytes seen at sites of inflammation have the potential to provide several thousand times higher concentrations of calprotectin.

DISCUSSION

An apparent threshold level of Ag(I) reduced membrane potential and membrane integrity rapidly for the individual cells of C. albicans and C. maltosa, suggesting that a major target of silver is located in the cell membrane. The absence of such a threshold dose for Hg(II) suggested that the target molecules and their threshold levels of mercury were different from those of silver. Moreover, in Ag(I) solutions, cells lost recoverability at a rate similar to those for cell depolarization and membrane permeabilization, whereas in Hg(II) solutions, loss of cell recoverability preceded the loss of membrane potential and membrane integrity, especially for C. maltosa. C. albicans retained membrane integrity even after exposure to Hg(II) for 1 h and in this regard differed significantly from C. maltosa. A further distinction between the activities of the two ions was the fact that the uptake and binding of Ag(I) by C. maltosa were greater and more rapid than those of Hg(II).

Brown and Smith (4) showed by a cytochemical method that the Hg(II) accumulated by Cryptococcus albidus was present in various parts of the cell other than the cell wall and membranes. Passow and Rothstein (16) demonstrated that mercury ions caused irreversible membrane damage in S. cerevisiae, whereas Brunker (5) found that this metal inactivated the enzymes that are responsible for catabolic metabolism. These reports suggested that mercury ions might interact with a variety of reactive sites in both the cell membrane and intracellular targets. An interaction of Hg(II) with C. albicans and C. maltosa at multiple sites with disruption of vital cell processes might explain the observed loss of cell recoverability before the loss of membrane potential and membrane integrity. Ag(I) and Hg(II) may act similarly for both yeasts and possibly with different targets, but the more-rapid binding of Ag(I) may overshadow any threshold differences between membrane function and cell recoverability.

We recognize that chemical forms of a metal in solution, which regulate metal binding to the membrane and penetration into the cell, are difficult to identify and vary under different experimental conditions (6, 12). Nevertheless, relative metal toxicity may be assessed from the equivalent biologically active metal concentrations. We found that the percentage of depolarized cells of both species increased with increasing concentrations of metals and generated a sigmoidal dose-response relationship. These sigmoidal curves permitted an estimation of metal concentrations that remained in the supernatants.

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