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Secretion of functional antibody and Fab fragment from yeast cells

(*Saccharomyces cerevisiae*/chimeric antibody/yeast signal sequence/tumor antigen binding/effector functions)

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ABSTRACT **We have constructed yeast strains that secrete functional mouse–human chimeric antibody and its Fab fragment into the culture medium. For chimeric whole antibody, cDNA copies of the chimeric light-chain and heavy-chain genes of an anti-tumor antibody were inserted into vectors containing the yeast phosphoglycerate kinase promoter, invertase signal sequence, and phosphoglycerate kinase polyadenylylation signal. Simultaneous expression of these genes in yeast resulted in secretion of properly folded and assembled chimeric antibody that bound to target cancer cells. Yeast chimeric antibody exhibited antibody-dependent cellular cytotoxicity activity but not complement-dependent cytotoxicity activity. For production of Fab fragments, a truncated heavy-chain (Fd) gene was created by introducing a stop codon near the codon for the amino acid at which papain digestion occurs. Simultaneous expression of the resulting chimeric Fd and light-chain genes in yeast resulted in secretion of properly folded and assembled Fab fragment that bound to target cancer cells.**

While a number of single-chain heterologous proteins have been secreted from yeast (1–7), the secretion of foreign multimeric or heterodimeric proteins has not been reported. Of the latter two groups, antibody molecules or the protein fragments that contain their antigen-binding domains, Fab and $F(ab')_2$ (Fig. 1), are particularly important for a wide variety of applications, including detection and treatment of human disease (8, 9), *in vitro* diagnostic tests (10), and affinity purification methods (11). Chimeric antibodies that consist of mouse variable (V) regions fused to human constant (C) regions may be especially valuable for human therapeutic or *in vivo* diagnostic uses, since they are potentially less immunogenic in humans than are mouse antibodies.

We have described (12) the development of a cDNA cloning strategy for the construction and expression of chimeric antibodies. In this approach, cDNAs coding for the mouse V regions are fused at the immunoglobulin joining (J) region to cDNAs coding for human IgG1 C regions. The cDNA approach for chimeric antibody construction provides an ideal starting point for expression of these genes in microbial systems that either do not undertake mRNA splicing or do so only rarely. In this paper, we describe the secretion from yeast of chimeric antibody and Fab protein. The yeast-secreted molecules (both whole antibody and Fab) bind to target cells as well as their lymphoid cell-derived counterparts. In addition, yeast-derived whole antibody has the same antibody-dependent cellular cytotoxicity (ADCC) activity observed with lymphoid cell-derived whole chimeric antibody but lacks the complement-dependent cytotoxicity (CDC) activity shown by the lymphoid cell-derived antibody.

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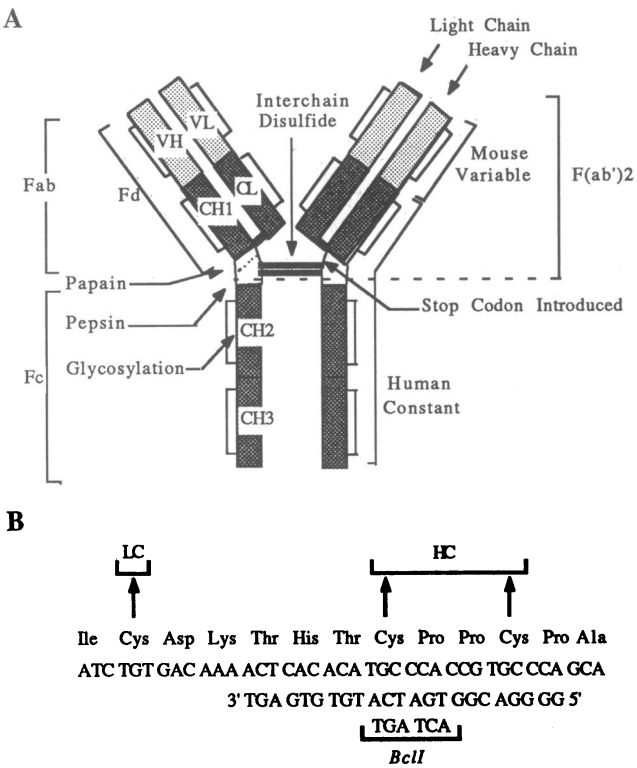

FIG. 1. (*A*) Structure of mouse–human chimeric IgG1. The locations of papain and pepsin cleavage sites and the structures of F(ab) and $F(ab')_2$ resulting from digestion with these enzymes are shown. The locations of N-linked glycosylation and a stop codon introduced by site-directed mutagenesis are also shown. V, variable; C, constant; H, heavy; L, light. (*B*) Site of *in vitro* mutagenesis and DNA sequence of the mutagenesis primer used to place a stop codon and *Bcl* I site in the sequence encoding the hinge region of human γ1 heavy chain. The *Bcl* I site was converted to *Xho* I by digestion with *Bcl* I followed by treatment with phage T4 DNA polymerase and ligation with *Xho* I linkers. The stop codon was unaffected by this treatment. Arrows indicate interchain disulfide bonds with light chain (LC) and heavy chain (HC).

MATERIALS AND METHODS

Strains and Media. *Escherichia coli* strain MC1061 (13) was used as a host for plasmids. *E. coli* strain 71.18 (14) was used as a host for bacteriophage M13. *Saccharomyces cerevisiae* strain BB331C (*MATa ura3 leu2*) was used as a host for yeast transformations performed as described by Ito *et al.* (15). *E. coli* was grown in TYE broth (1.5% Tryptone/1.0% yeast extract/0.5% NaCl) or agar (1.5% Bacto) supplemented, as

Abbreviations: PGK, phosphoglycerate kinase; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; C, constant; V, variable.

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needed, with ampicillin (50 μg/ml). Yeast transformants were selected on SD agar (2% glucose/0.67% yeast nitrogen base/2% agar) and grown in SD broth buffered with 50 mM sodium succinate (pH 5.5).

***In Vitro* Mutagenesis.** Site-directed *in vitro* mutagenesis to place restriction sites at yeast or mammalian signal-sequence processing sites and a stop codon in the heavy-chain hinge region was performed as described by Kramer *et al.* (14). Phage plaques containing the desired mutation were identified by plaque-filter hybridization with ^{32}P-labeled primer.

Enzyme-Linked Immunosorbent Assay (ELISA). Light chain was detected by double-antibody sandwich ELISA (16) using goat anti-human κ antiserum as the coating antibody and peroxidase-labeled goat anti-human κ antiserum for quantitation of bound κ protein. Heavy chain was detected similarly with goat anti-human γ antisera with and without peroxidase label. Association of κ light chains and γ heavy chains was detected with goat anti-human κ antiserum as coating antibody and peroxidase-labeled goat anti-human γ antiserum as the second antibody.

Isolation of Chimeric Whole Antibody and Chimeric Fab from Yeast. Whole antibody was purified from the culture supernatant of a 10-liter fermentation as follows. The culture supernatant was concentrated by ultrafiltration (DC10 ultrafiltration system with spiral cartridge, 30-kDa size cutoff; Amicon), filtered through a 0.45-μm filter, and concentrated over a YM30 filter (Amicon) to 250 ml. Antibody protein was purified from the concentrated supernatant by protein A–Sepharose chromatography (11). Analysis of this protein by nonreducing polyacrylamide gel electrophoresis followed by Coomassie blue staining and immunoblotting with anti-human κ antiserum (Sigma) as probe revealed a whole immunoglobulin-size protein band against a background smear. The protein in this band was purified by HPLC on an AB_x (5-μm particle size) column (Baker), with elution by a linear gradient of 10–125 mM potassium phosphate (pH 6.8).

Fab protein was purified from 1 liter of culture supernatant by concentrating over an Amicon YM30 filter, washing with 130 ml of 10 mM potassium phosphate at pH 7.5 (buffer A), and reconcentrating to 12.5 ml. The supernatant was diluted to 54 ml with buffer A and loaded onto a 1.5-ml S-Sepharose column, washed with 20 ml of buffer A, and eluted with a 40-ml linear gradient of 0–200 mM NaCl in buffer A.

Fab protein prepared by papain digestion (11) of 3 mg of whole L6 mouse antibody or chimeric antibody was purified on a 25-ml S-Sepharose column by elution with an 80-ml linear gradient of 0–120 mM NaCl prepared in 10 mM sodium phosphate (pH 7.5). The Fab protein was eluted at 60 mM NaCl and was free of Fc protein.

Functional Tests of Chimeric Antibody and Fab from Yeast. The following tests were used to assess function: (*i*) direct binding of whole antibody or Fab to target cells that are positive or negative for the L6 antigen; (*ii*) competition inhibition of binding of L6 mouse antibody to antigen-positive cells; (*iii*) ADCC and CDC assays with whole antibody. The binding assays were performed with a Coulter model EPIC-C cell sorter (17). ADCC and CDC assays were performed with ^{51}Cr-labeled target cells (18, 19) that were exposed to the antibodies and peripheral blood leukocytes or human serum over a period of 4 hr.

RESULTS

Construction of Yeast Expression Plasmids Containing Antibody Genes. To facilitate light- and heavy-chain secretion from yeast, the gene sequences encoding the mature forms of the light and heavy chains of a chimeric anti-tumor antibody (L6, ref. 12) were fused to the yeast invertase signal sequence (20) and placed under the control of the phosphoglycerate kinase (PGK) gene promoter (21). These fusions were then cloned into yeast expression vectors containing the PGK polyadenylylation signal (21) to generate pING1441 (light chain, *leu2*) and pING1442 (heavy chain, *ura3*) (Fig. 2*A*).

The Fd portion of heavy chain consists of the V region and the C_{H1} domain (Fig. 1) and is generated by digestion of whole antibody with papain (11). To enable yeast to synthesize Fd protein, a stop codon was introduced by site-directed mutagenesis of the chimeric heavy-chain gene so that translation would terminate in the hinge region, near the papain recognition site (Fig. 1*B*). A *Bcl* I site was introduced along

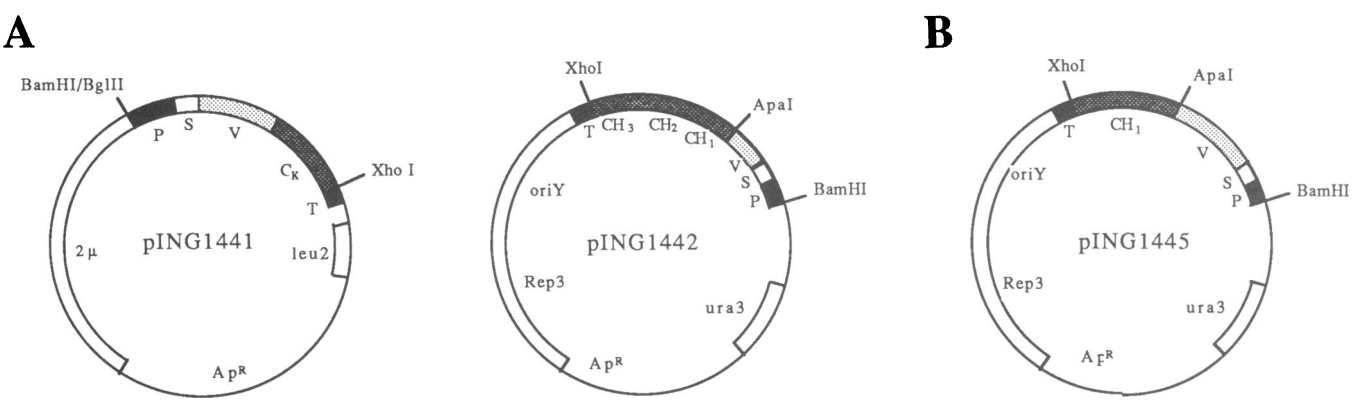

FIG. 2. Structure of yeast immunoglobulin expression plasmids. The fusions of the gene sequences encoding the mature forms of light and heavy chain to the yeast invertase signal sequence and PGK promoter were accomplished by first introducing by *in vitro* mutagenesis a unique restriction site at the signal-sequence processing sites for both the invertase signal sequence (*Pst* I) and the light (*Aat* II) and heavy (*Sst* I) chain genes. These sites were positioned such that a blunt-ended ligation of restriction enzyme-digested, T4 DNA polymerase-treated DNAs resulted in in-phase translational fusions of the 5′ end of the mature immunoglobulin chains with the 3′ end of the yeast invertase signal sequence. (*A*) The light-chain expression plasmid, a pING1441, was constructed by cloning a *Bgl* II–*Xho* I fragment containing the light-chain gene into a yeast expression vector, pING804CVS (provided by J.-H. Lee, International Genetic Engineering). pING1441 contains the complete 2-μm plasmid (2 μ); the chimeric light-chain gene (V and $C_κ$ regions) fused to the PGK promoter (P), invertase signal sequence (S), and PGK transcription termination and polyadenylylation signals (T); and the *leu2* gene as the yeast selective marker. The heavy-chain expression plasmid, pING1442, was constructed by cloning a *Bam*HI–*Xho* I fragment containing the heavy-chain gene into a yeast expression vector, pING1150. pING1442 contains the yeast origin of replication (*oriY*) and a cis-acting stabilization sequence (Rep3) from yeast 2-μm plasmid; the chimeric heavy-chain gene (V-region and C-region domains C_{H1}, C_{H2}, and C_{H3}) fused to the PGK promoter, invertase signal sequence, and PGK transcription termination and polyadenylylation signals; and the *ura3* gene as the yeast selective marker. (*B*) The Fd-chain expression plasmid, pING1445, is identical to the heavy-chain expression plasmid, pING1442, with the exception that the *Apa* I–*Xho* I restriction fragment in pING1442 encoding C_{H1}, C_{H2}, and C_{H3} has been replaced with a fragment encoding only C_{H1}.

with the stop codon. Following conversion of the *Bcl* I site to *Xho* I, the *Apa* I–*Xho* I C_H-region fragment of pING1442 (Fig. 2*A*) was replaced with an *Apa* I–*Xho* I C_{H1} fragment containing the stop codon in the hinge region to generate pING1445 (Fig. 2*B*).

Secretion of Whole Chimeric Antibody from Yeast. The plasmids pING1441 and pING1442 were cotransformed into *S. cerevisiae* strain BB331C by selection for Ura^+ Leu^+ colonies. Ten transformants were grown for 3 days in 5 ml of SD broth and the culture supernatants were analyzed by ELISA for the levels of light chain, heavy chain, and associated light and heavy chains. The culture supernatants of two transformants (nos. 1 and 5) contained light chain at ≈100 ng/ml and heavy chain at 50–80 ng/ml, and 50–70% of the heavy chain was associated with light chain. These proteins were concentrated on a Centricon 30 filter (Amicon), electrophoresed in a $NaDodSO_4$/7% polyacrylamide gel under nonreducing conditions, and transferred to nitrocellulose. κ-crossreactive protein was detected with goat anti-human κ antiserum followed by peroxidase-labeled rabbit anti-goat antiserum. A faint, but distinct, band that comigrated with purified chimeric L6 antibody produced by Sp2/0 cells was observed in the lanes containing both supernatants (data not shown). These results suggested that the yeast transformants were synthesizing and secreting a fully assembled chimeric antibody that was very similar to lymphoid cell-derived antibody.

To prepare sufficient quantities of yeast-derived antibody for detailed characterization of structure and function, transformant no. 5 was grown in a 10-liter fermentor for 58 hr. Whole antibody was purified from this culture medium as described in *Materials and Methods*. ELISA analysis of column fractions from HPLC (AB_x 5-μm) revealed a heavy (γ) and light (κ) chain-crossreactive peak corresponding to a distinct A_{280} peak. Analysis of these fractions by nonreducing $NaDodSO_4$/polyacrylamide gel electrophoresis revealed a protein that comigrated with L6 chimeric antibody purified from Sp2/0 cells (Fig. 3). Under reducing conditions, the protein in these fractions was resolved into a light-chain band, which comigrated with the light chain of L6 chimeric antibody from Sp2/0 cells, and two heavy chain bands, which migrated near the heavy chain from Sp2/0 cells (Fig. 3). This purified preparation of yeast-produced chimeric antibody was used in further binding and function assays.

Secretion of Chimeric Fab from Yeast. The plasmids pING1441 (Fig. 2*A*) and pING1445 (Fig. 2*B*) were cotransformed into *S. cerevisiae* BB331C and the transformants were grown in broth under selective conditions as described above. The culture supernatants were assayed by ELISA and were found to contain light chain at 100–200 ng/ml. To determine whether the cells secreted a Fab-size protein, the culture supernatants were concentrated with Centricon 30 filters, electrophoresed in a nonreducing $NaDodSO_4$/10% polyacrylamide gel, and electrophoretically transferred to nitrocellulose paper. Light chain-crossreactive protein was detected with goat anti-human κ antiserum followed by peroxidase-labeled rabbit anti-goat antiserum. One of the five transformants secreted a distinct κ-crossreactive band, which migrated at the expected position for Fab protein (data not shown).

The yeast strain that secreted the Fab-size protein was grown in 1 liter of SD broth for 4 days at 30°C and Fab protein was purified from the culture supernatant. Nonreducing $NaDodSO_4$/polyacrylamide gel analysis of pooled S-Sepharose column fractions containing anti-human κ-crossreactive protein revealed a 46-kDa protein, comigrating with chimeric Fab prepared by papain digestion of Sp2/0 cell-derived chimeric whole antibody (Fig. 4). Electrophoresis under reducing conditions resolved this protein into two bands that migrated at 23 and 25 kDa (Fig. 4). The 23-kDa band was identified as light chain by its reactivity on an immunoblot with anti-human κ antiserum. These results were consistent with the predicted molecular masses, based on nucleotide sequence, for fully processed L6 chimeric light chain (23.3 kDa) and Fd chain (24.8 kDa).

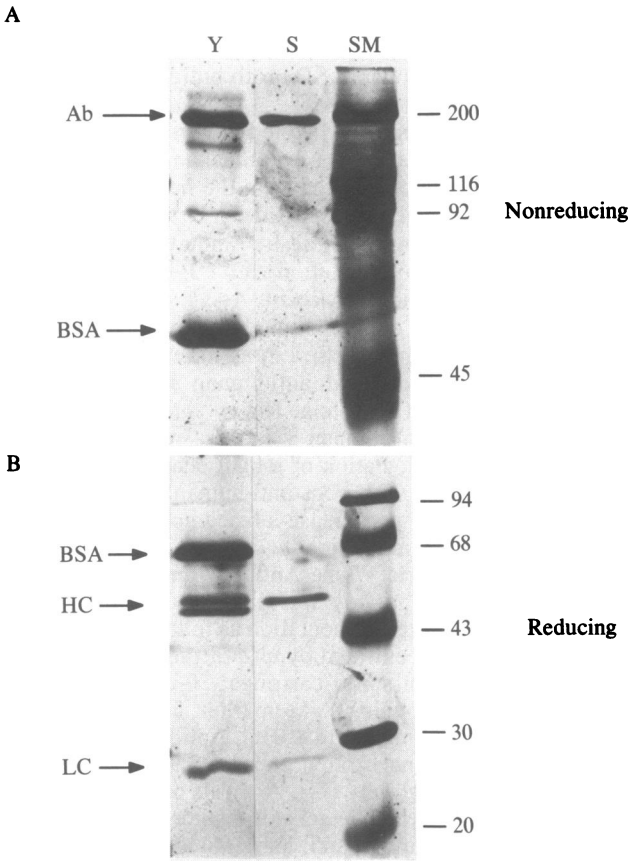

FIG. 3. Silver-stained $NaDodSO_4$/polyacrylamide gels showing purified chimeric antibody secreted by yeast. (*A*) Nonreducing 7% gel. (*B*) Reducing 10% gel. Intensely stained band at 68 kDa on both gels is bovine serum albumin (BSA), which was present as a carrier protein. Size marker (lanes SM) molecular masses (in kDa at right) and relevant yeast-derived (lanes Y) and Sp2/0-derived (lanes S) antibody bands [Ab on nonreducing gel; HC (heavy chain) and LC (light chain) on reducing gel] are identified.

Binding Characteristics of Chimeric Whole Antibody and Fab Protein Secreted by Yeast. The purification from yeast culture supernatants of protein of the expected size of whole antibody and Fab suggested that the yeast transformants were secreting correctly folded, functional molecules. This hypothesis was confirmed by performing direct and competition binding assays with a human colon carcinoma cell line (line C-3347) that expresses 5×10^5 molecules of the L6 tumor antigen per cell (17). In the direct binding assay, both whole antibody and Fab from yeast bound to the target cancer cells but not to a control cell line that lacked the L6 antigen (data not shown). In the competition assay using mouse L6 antibody, 50% binding inhibition was observed at the same concentration (2 μg/ml) for both yeast-derived and Sp2/0 cell-derived whole chimeric antibody (Fig. 5). In the same assay, the yeast-derived L6 chimeric Fab behaved identically to both Sp2/0 cell-derived chimeric and mouse Fab proteins prepared by papain digestion (Fig. 5). Fifty percent inhibition of mouse L6 antibody was achieved by the yeast-derived Fab at 7 μg/ml.

Chimeric Whole Antibody from Yeast Mediates ADCC but Not CDC. Two additional tests for function were performed with chimeric whole antibody from yeast: (*i*) mediation of ADCC in the presence of human peripheral blood leukocytes

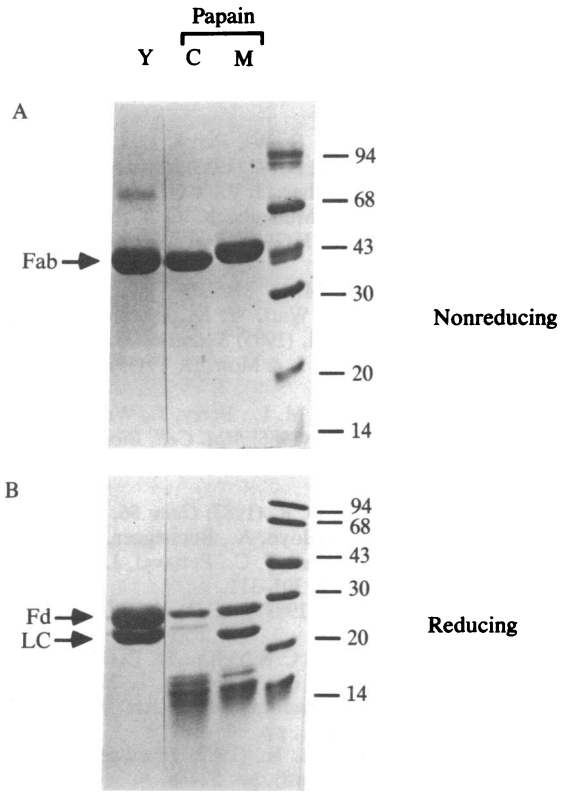

FIG. 4. Coomassie blue-stained gel comparing purified chimeric Fab protein secreted by yeast (lanes Y) with chimeric (lanes C) and mouse (lanes M) Fab fragments produced by papain digestion of intact antibody. (*A*) Nonreducing 10% gel. (*B*) Reducing 12% gel. Size marker molecular masses (kDa) and relevant bands are shown. LC, light chain.

and (*ii*) mediation of tumor-cell lysis in the presence of human complement. ADCC activity of yeast-derived chimeric L6 antibody was slightly higher than that of Sp2/0 cell-derived chimeric antibody, and the ADCC activities of both chimeric antibodies were higher than that of the mouse L6 antibody (Table 1). Yeast-derived L6 chimeric antibody failed to mediate CDC, even at the highest antibody concentration, whereas Sp2/0 cell-derived chimeric L6 antibody and mouse L6 antibody exhibited the expected cytolytic activity (Table 2).

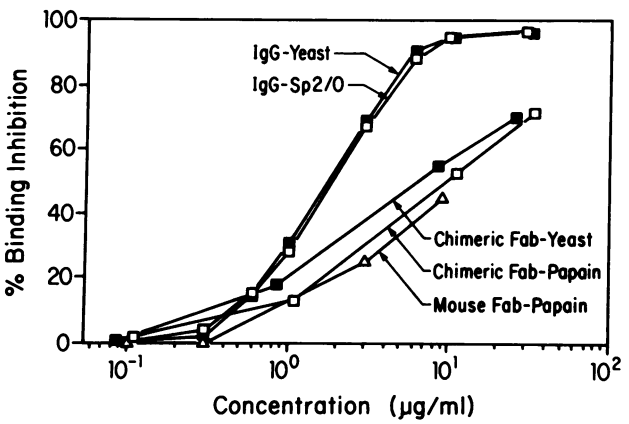

FIG. 5. Comparison in antibody-competition binding assays between whole chimeric L6 antibody (IgG) derived from yeast and Sp2/0 cells, chimeric L6 Fab derived from yeast or prepared by papain digestion of whole chimeric antibody isolated from Sp2/0 cells and mouse Fab prepared by papain digestion of L6 antibody. C-3347 colon carcinoma cells were incubated with various concentrations of unlabeled blocking antibodies before addition of fluorescein isothiocyanate-conjugated mouse L6 antibody (3 μg/ml). Inhibition was measured by flow cytofluorimetry.

Table 1. ADCC analysis

Antibody	Conc., μg/ml	% cytolysis
Standard mouse L6	5.0	42
	1.0	48
Sp2/0 chimeric L6	1.0	96
	0.1	71
	0.01	54
	0.001	37
Yeast chimeric L6	1.0	114
	0.1	108
	0.01	76
	0.001	60
None	0	23

The colon carcinoma target cells (line C-3347) were labeled with ^{51}Cr and exposed for 4 hr to a combination of monoclonal antibody and human peripheral blood leukocytes (100 per target cell), and the release of ^{51}Cr was measured subsequently. The release of ^{51}Cr (after corrections of values for spontaneous release from untreated cells) is a measure of cytolysis.

DISCUSSION

We have engineered the yeast *S. cerevisiae* to secrete functional mouse–human chimeric antibody and Fab protein into the culture medium. This was accomplished by simultaneous expression of the mature light-chain gene and the heavy-chain gene or a truncated heavy-chain (Fd) gene fused to the yeast invertase signal sequence and the PGK promoter and polyadenylylation signal. Several lines of evidence support the thesis that these proteins are correctly folded. (*i*) Proteins of the expected size for whole antibody and Fab were purified from the culture supernatants of yeast cells expressing the chimeric light- and heavy- or Fd-chain genes (Figs. 3 and 4). (*ii*) The whole antibody and Fab from yeast behaved indistinguishably from their lymphoid cell-derived counterparts in both direct and competition binding assays (Fig. 5). (*iii*) The chimeric whole antibody from yeast exhibited the same ADCC activity as the chimeric antibody from Sp2/0 cells (Table 1).

There have been a number of reports of secretion from yeast of heterologous proteins fused to yeast signal sequences (2, 4, 5, 7). All of the proteins in these examples were composed of a single polypeptide chain. Although there is one example of functional mouse antibody (IgM) production in yeast, the antibody in this case was found only intracellularly in vacuoles; only unassociated light and heavy chains were detected in the culture supernatant (22).

Table 2. CDC analysis

Antibody	Conc., μg/ml	Complement*	% cytolysis†
Standard mouse L6	5	+	122
	1	+	53
	5	−	1
Sp2/0 chimeric L6	5	+	73
	1	+	22
	0.1	+	5
	5	−	2
Yeast chimeric L6	5	+	3
	1	+	2
	0.1	+	4

*Human serum from a healthy subject was used as the source of complement.

†^{51}Cr-labeled C-3347 cells were exposed to human complement and antibody. CDC was measured by a 4-hr ^{51}Cr-release assay.

The chimeric whole antibody from yeast mediated ADCC in an identical fashion to Sp2/0 cell-derived chimeric antibody (Table 1). These results suggest that the yeast-derived antibody is equivalent to the lymphoid cell-derived antibody in interacting with Fc receptors on killer cells and activating them to mediate ADCC. Interestingly, the chimeric mouse–human antibody from either lymphoid cells or yeast was more efficient at ADCC than was the mouse antibody. Thus, the Fc–receptor interaction involved in ADCC appears to be primarily determined by the amino acid sequence of the Fc portion of the antibody and is probably not affected by the altered glycosylation patterns expected for yeast-derived antibody. This conclusion is consistent with the observation that binding of Fc receptor type I by IgG, which may play a central role in ADCC (23), occurs in the region linking the C_{H2} domain to the hinge (24). This region of the protein is an exposed, flexible strand (24) and is well removed from the site of N-linked glycosylation (25).

An intriguing result is that the yeast-derived antibody lacks the ability to activate complement to lyse target cells (Table 2). This may reflect differences in the glycosylation patterns of the yeast-derived and Sp2/0 cell-derived chimeric antibodies. Several observations support this hypothesis. Binding of complement component C1q, which initiates CDC, occurs within the C_H2 domain of human IgG (26), which is also the region of N-linked glycosylation. Second, elimination of the N-linked glycosylation site in mouse IgG2b by *in vitro* mutagenesis of asparagine-297 to alanine results in reduced affinity of the antibody for human C1q and complete loss of CDC (27). Third, while yeast and mammalian cells recognize the same peptide signal [Asn-Xaa-(Ser/Thr)] for glycosylation and utilize a similar pathway for core oligosaccharide synthesis in the endoplasmic reticulum (28), the type and extent of outer chain glycosylation appear to be quite different in these cell types (29). Indeed, comparison of yeast- and Sp2/0 cell-derived heavy chains by reducing NaDodSO$_4$/polyacrylamide gel electrophoresis revealed differences in protein mobility (Fig. 4), which may be caused by differences in glycosylation. Such glycosylation differences may be sufficient to cause a loss of CDC activity. Further studies will be required to establish the exact cause of these size differences and their possible relationship to CDC activity.

Fab proteins may be especially useful for certain diagnostic and therapeutic applications, including *in vivo* tumor imaging (8) and drug or toxin delivery to tumors. Mouse–human chimeric Fab proteins are particularly attractive for these uses because they may be less immunogenic than mouse Fab proteins in humans. Current methods for Fab production involve papain digestion of purified whole antibody (11). This approach can be problematic, since not all antibodies are equivalent in their susceptibility to papain cleavage (11) and additional purification steps are required beyond that for the whole antibody. In addition, papain digestion can result in partial degradation of the Fab (Fig. 4). By contrast, direct production of Fab by yeast or, as described elsewhere, by *E. coli* or Sp2/0 cells (30) can yield a highly purified, homogeneous Fab preparation that has the same binding activity as the Fab prepared by papain digestion (Fig. 5). Further, the introduction by site-directed mutagenesis of a restriction site in conjunction with the stop codon in the Fd sequence permits manipulations at the 3′ end of the gene that can yield Fab proteins with altered properties, such as enhanced affinity in labeling reactions, or allow direct production of Fab molecules linked to various proteins.

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