BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 856–860 (1997)
ARTICLE NO. RC977402

Characterization of Recombinant Bovine Conglutinin Expressed in a Mammalian Cell

Yasuhiko Suzuki,* Souji Eda,* Takao Kawai,† Katsuki Ohtani,* Tetsuo Kase,‡ Takashi Sakamoto,§ and Nobutaka Wakamiya§,[1]

**Department of Pathology, †Department of Food Microbiology, and ‡Department of Virology, Osaka Prefectural Institute of Public Health, Higashinari, Osaka 537, Japan; and §Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan*

Received August 25, 1997

We describe here the successful expression of recombinant bovine conglutinin in CHO cells as well as its physical and biological characteristics. Geneticin-resistant transformants harboring bovine conglutinin cDNA in the expression vector pNOW/CMV-A were screened by Western blot analysis for secretion into media of recombinant conglutinin. A four-day amplification of the transgene with increasing concentrations of methotrexate resulted in a dose-dependent increase in the production of recombinant conglutinin to a final concentration of 18.6 μg/ml of media. Recombinant conglutinin purified from this media by affinity column chromatography on mannan-agarose had a migration pattern similar to that of native conglutinin on polyacrylamide gel electrophoresis under reducing, nonreducing, and native conditions. The recombinant conglutinin exhibited sugar binding, conglutination, hemagglutination inhibition, and neutralization of influenza A virus, activities engaged in by the native conglutinin. This is the first report describing a high level of expression of a serum cruciform collectin with the full range of biological activity.

Conglutinin is a bovine serum protein which was originally characterized as a molecule that mediates conglutination (1). This lectin was also the first member of the vertebrate C-type lectin family to be described (2), and recently, conglutinin has been classified as a member of a subfamily of vertebrate C-type lectins termed called collectins which carry a collagen-like domain (3). Conglutination is a biological activity which is known to occur through the binding of conglutinin to the carbohydrate moiety on the alpha chain of iC3b (4, 5). This activity is inhibited by certain chelating agents such as EDTA. The binding of conglutinin to yeast cell extracts has also been found to be calcium-dependent (6) and can be inhibited by various sugars, with the strongest inhibition noted with N-acetyl-D-glucosamine (7). Much attention has been currently devoted to mammalian lectins, focussing on their role in innate immunity involving complement activation or immunoglobulin-independent defense by opsonization (8). In our recent experiments, conglutinin was found to inhibit both infection and hemagglutination by influenza A virus (9). Evidence that conglutinin acts as an opsonin for influenza A virus has been presented by Hartshorn et al. (10). Further investigation into the biological activities of conglutinin appears to require large amounts of this lectin. Therefore, analysis of conglutinin at the molecular level and its expression as a recombinant protein were attempted.

The cDNA encoding conglutinin has been cloned and sequenced (11, 12, 13), and the liver was shown to be the predominant organ expressing the mRNA. Three groups have characterized the recombinant conglutinin expressed in *E. coli* and all described the neck and carbohydrate recognition domains of this molecule (14, 15, 16). This recombinant conglutinin showed a binding specificity which was similar to that of native conglutinin. It also unexpectedly inhibited hemagglutination by influenza A virus in a calcium dependent manner, even though it had 20 times less conglutination activity than that of the native protein (14) and no neutralizing activity for influenza A virus (unpublished data). These results suggested that the entire structure of conglutinin is essential for display of its full activity.

In order to investigate the complete biological activity of conglutinin, we expressed the entire molecule in a

[1] To whom correspondence should be addressed. Fax: (81-6) 875-3894. E-mail: wakamiya@biken.osaka-u.ac.jp.

Abbreviations used: Bkg, bovine conglutinin; BKg-CRD, bovine conglutinin composed of carbohydrate recognition domain; Gal, galactose; Glc, glucose; GlcNac, *N*-acetyl-D-glucosamine; Mal, maltose; Man, mannose; VC, vitamin C.

0006-291X/97 $25.00

mammalian expression system and characterized this recombinant form in terms of structure, biochemistry and biological activity.

MATERIALS AND METHODS

Construction of an expression vector and expression of conglutinin in CHO cells. The 1.5 kbp cDNA for conglutinin, which extends from 100 bp upstream of the initiation codon to 170 bp downstream of the stop codon, was amplified using the polymerase chain reaction (PCR) method with the primers 5′-AAGCGGCCGCTCTCAACTTGC-TTTCCTGTG-3′ and 5′-CCTCTAGAGCCAGTTTCAAACTTTATTC-3′. The PCR was performed in a TP-cycler (Toyobo Co.), and consisted of 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for extension, followed by 5 min at 72°C for the final extension. The PCR product was digested with the restriction endonucleases *Not* I and *Xba* I and cloned into the expression vector pNOW/CMV-A (construction to be presented elsewhere) which was then designated pNOW-BKg. The transfection of CHO DG44 was accomplished using a DOTAP (Boehringer Mannheim GmbH) transfection reagent. The cells were plated at a density of 2×10^5 cells/6 ml in 60 mm plastic culture dishes in Iscove's modified Dulbeco's Medium (I-MDM) supplemented with 100 mM hypoxanthine, 10 mM thymidine and 10 % FCS for 20 h prior to transfection and were then transfected with the plasmid pNOW-BKg complexed with DOTAP. Geneticin-resistant (400 μg/ml) transfectants were identified at 2 weeks and selected colonies were subcloned in a 96-well microtiter plate. The transformed CHO DG44 cell line with the highest level of production of conglutinin was subjected to increasing concentrations of MTX 10 nM followed by treatment with 50 nM in I-MDM supplemented with 10 % FCS and 400 μg/ml G 418. Cells after selection of MTX were cultured with I-MDM containing 10% FCS and vitamin C of 50 μg/ml. The recombinant conglutinin content of tissue culture media was determined by a Western blotting technique using anti-sera raised in a New Zealand White rabbit by serial injection of purified native conglutinin. Membranes containing adsorbed proteins were sequentially incubated with anti-conglutinin serum, biotinylated anti rabbit-IgG and alkaline phosphatase conjugated streptavidin. Immunoreactive species were visualized with nitroblue tetrazorium chloride (NBT) and 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt (BCIP). The lower limit of sensitivity for the assay was approx. 50 ng/ml and the intensity was linear over a range of 50-1000 ng/ml.

Purification of native and recombinant conglutinin and biochemical characterization. Native conglutinin was isolated from bovine serum as described by Haurum et al. (17). Briefly, conglutinin in heat-inactivated (56°C, 30 min) bovine serum or in culture supernatants of stable transformants was precipitated with 4% (w/v) polyethylene glycol-6000. The precipitate was redissolved in TBS-Ca (10 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and passed through a column of mannan-agarose (Sigma, U.S.A.). The column was washed with TBS-Ca and proteins were eluted with TBS-E (10 mM Tris, 150 mM NaCl, 10 mM EDTA). The EDTA eluate was recalcified to 10 mM with $CaCl_2$ and re-applied to the mannan-agarose column. Pure conglutinin was eluted from the mannan-agarose with 2 mM N-acetyl-D-glucosamine. The fractions containing conglutinin were pooled and used for further studies. Protein samples treated with sodium dodecyl sulfate (SDS), with SDS and 2-mercaptoethanol, or left untreated were separated by polyacrylamide gel electrophoresis and detected by staining with Coomassie Brilliant Blue R250 or by immunostaining with rabbit anti-conglutinin serum as described above. Solid-phase saccharine binding assays were performed using PV-mannose, a synthetic polymer composed of vinylbenzyl monomer coupled to mannose (Seikagaku Co., Japan). Briefly, microtiter plates were coated with PV-mannose at a concentration of 10 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6) by overnight incubation at 4°C. TTBS-Ca (10 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween 20, pH 7.4) was used in all washing steps. After washing, the plates were blocked with Block Ace (a blocking solution made from skim milk, purchased from Dainippon Pharmaceuticals Co.) and washed again. The plates were then incubated at 37°C for 1 hour with samples diluted with TTBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) with or without $CaCl_2$ or EDTA at a concentration of 5 mM. The plates were washed with appropriate buffer and incubated with rabbit anti-recombinant conglutinin-CRD IgG diluted 1/2000. After washing, the plates were incubated at 37°C for 1 hour with biotinylated anti-rabbit IgG diluted 1/1000 and at 37°C for 30 min with horseradish peroxidase conjugated streptavidin diluted 1/10000. After a final washing, the bound enzymatic activity was estimated by adding 100 μl TMB-peroxidase substrate (Kirkegaard & Perry Laboratories, USA). The absorbance of the wells was read with Microplate reader (Bio-Rad Laboratories, USA) at 450 nm after stopping the reaction by adding 100 μl of phosphoric acid.

Amino acid analysis. Proteins for amino acid analysis were run on SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane prior to detection with Ponceau-S dye. Protein bands were excised and amino acid analysis was performed with the Waters Pico-Tag system.

Assay of biological activities. Conglutination of native and recombinant conglutinin was measured as described by Wakamiya et al. (9). Conglutination activity was detected by a 37°C incubation in veronal buffer of 50 μl of the native or recombinant conglutinin and 1 % sheep red blood cells (SRBCs) harboring iC3b, followed by an examination of agglutination. Hemagglutination inhibition (HI) tests were performed using a standard microtiter assay method (18). Inhibition of influenza A virus-mediated agglutination of chicken erythrocytes by native and recombinant conglutinin was observed after a 1-hour incubation at room temperature in 96-well microtiter plates in the presence of 1 % (v/v) chicken erythrocytes. A rapid neutralization test for influenza virus was performed as described by Okuno et al. (18). Ninety-six well microtiter plates were used for preparation of monolayers of Madin-Darby canine kidney (MDCK) cells and the peroxidase-antiperoxidase staining technique was used for detection of foci infected with influenza virus. The neutralization titer was expressed as 50% or less of the control value.

RESULTS

Transformation and amplification of the cDNA for conglutinin and purification of recombinant conglutinin. The expression vector pNOW/CMV-A used for this experiment contains the cytomegalovirus strong promoter which is adjacent to the polylinker *Hind* III-*Not* I-*Xba* I-*Apa* I, aph gene for selection by G418 and the dihydrofolate reductase gene for gene amplification. The CHO DG44 cell line was used for the expression of recombinant conglutinin. Cells were grown in 48 wells of ten 96-well microtiter plates seeded with 3×10^3 cells/well. Cells of 5 out of 21 recombinant conglutinin-producing wells with the highest production were cloned. The levels of recombinant conglutinin secreted by these lines ranged from 2.3-6.8 μg/ml after 4 days of culture. The 11A17 cell line exhibited the highest level which was 6.8 μg/ml after the 4-day culture. The 11A17 cell line was then subjected to selection with increasing concentrations of MTX to 10 nM followed by a 50 nM dose.

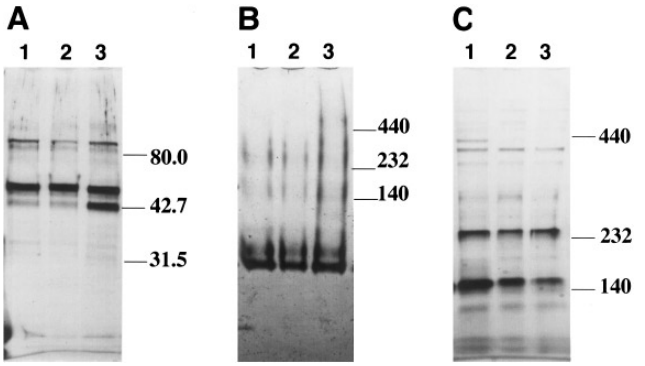

FIG. 1. SDS-polyacrylamide analysis of native and recombinant conglutinin. (A) Samples were treated with SDS and 2-mercaptoethanol and loaded onto a 10–20% polyacrylamide gel. (B) Samples were treated with SDS and loaded onto a 4–20% polyacrylamide gel. (C) Samples were directly loaded onto a 2–15% polyacrylamide gel. Lane 1, recombinant conglutinin produced in the presence of vitamin C (VC+); lane 2, recombinant conglutinin produced without vitamin C (VC−); lane 3, native conglutinin purified from bovine serum.

This selection resulted in a three fold increase in the concentration of recombinant conglutinin to a level of 18.6 μg/ml. The recombinant conglutinin was isolated from the culture medium of the MTX-amplified 11A17 line using affinity chromatography on mannan-agarose. We generally obtained more than 300 μg of recombinant conglutinin from 20 ml of media by mannan-agarose column chromatography.

Biochemical characterization of recombinant conglutinin. Polyacrylamide gel electrophoresis (PAGE) of the native and recombinant conglutinin under reducing conditions revealed a single band with a molecular mass of 45 kDa (Fig. 1A). This molecular species was identified as conglutinin by immunoblot analysis (data not shown). Both N-terminal amino acid sequence of 42.7 kDa-protein in native BKg (major band) and recombinant BKg (minor band) showed that they were the same partial fragment of BKg. The band of 42.7 kDa in native BKg was from auto-digested BKg during purification procedure which was previously described by Kawasaki (19) and Lu (20). Native and recombinant conglutinin were also analyzed by non-reducing PAGE (Fig. 1B) and by native PAGE (Fig. 1C) and both were found to have almost the same oligomeric structure. Amino acid analysis of the acid hydrolysate of the native and recombinant conglutinin showed post-translational hydroxylation of proline and lysine which was dependent on the presence of vitamin C (Table 1). The binding to PV-mannose of recombinant conglutinin produced in the presence or absence of vitamin C was inhibited by N-acetyl-D-glucosamine in a dose-dependent manner and the kinetics were almost identical (Fig. 2). The sugar binding specificity of recombinant conglutinin produced in the presence of vitamin C was similar to that of native conglutinin, and this was demonstrated with 4 other sugars as well (Table 2). Similar results were obtained with recombinant conglutinin produced in the absence of vitamin C (Data not shown).

Biological activities of recombinant conglutinin. Biological activities of native and recombinant conglutinin were examined using a microtiter plate assay system. Conglutination by both native and recombinant conglutinin was observed at concentrations of 20 and 40 ng/ml, respectively (Fig. 3A). The two forms of conglutinin exhibited identical specific activities in both neutralization tests (Fig. 3B) and HI tests (Fig. 3C). The minimum concentration required for inhibition of hemagglutination by H3N2 type influenza A virus was 20 ng/ml. Furthermore, the infectivity of H3N2 type virus was reduced to one half at a concentration of 56 ng/ml.

DISCUSSION

Functional studies on the carbohydrate recognition domains of mannose-binding protein and other C-type lectins have been successfully carried out following expression of these domains in *Escherichia coli.* However, there are no data concerning the expression in *E. coli*

TABLE 1

Amino Acid Composition of Native and Recombinant Conglutinin

	Native BKg		Recombinant BKg	
	cDNA[a]	This work[b]	−VC[b]	+VC[b]
Asx	27	27.4	26.8	28.6
Thr	15	14.8	15.5	14.7
Ser	22	23.0	20.2	22.3
Glx	40	44.8	41.4	43.8
Pro	40[c]	26.5	21.8	22.2
HyPro		8.1	6.3	8.7
Gly	67	66.0	58.0	63.8
Ala	28	28.0	28.0	28.0
Val	16	15.5	17.4	17.6
Met	6	5.5	5.3	4.8
Ile	10	9.6	11.4	11.6
Leu	17	17.6	21.0	20.8
Tyr	5	5.2	6.8	6.6
Phe	10	9.8	8.9	9.1
His	3	4.5	5.2	5.4
Lys	20[d]	15.0	19.5	17.9
HyLys		7.3	5.0	6.0
Arg	15	12.9	17.5	16.9
Cys	7	6.6	6.1	6.5

Note. The value shows the numbers of amino acid residues. Abbreviations: BKg, bovine conglutinin; VC, vitamin C.

[a] Calculated from the predicted amino acid sequence of conglutinin deduced from the cDNA sequence (14).

[b] Determined by the amino acid analysis of purified conglutinin.

[c] Total of Pro and HyPro.

[d] Total of Lys and HyLys.

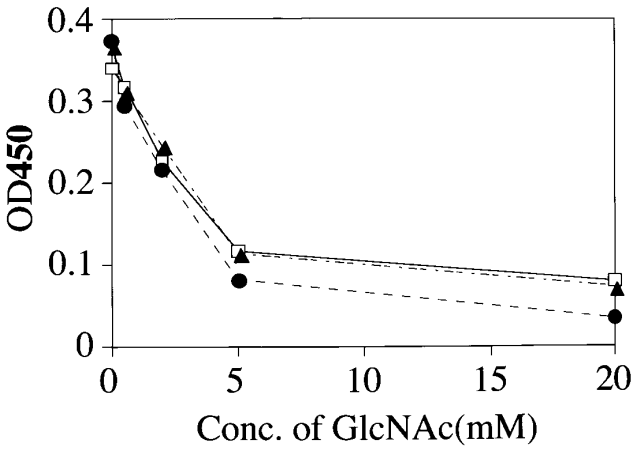

FIG. 2. Inhibition of binding of recombinant and native conglutinin to PV-mannose by N-acetyl-D-glucosamine. Inhibition test of binding of recombinant and native conglutinin to PV-mannose by various concentrations of N-acetyl-D-glucosamine was performed by ELISA. (▲) recombinant conglutinin VC+; (●) recombinant conglutinin VC−; (□) native conglutinin.

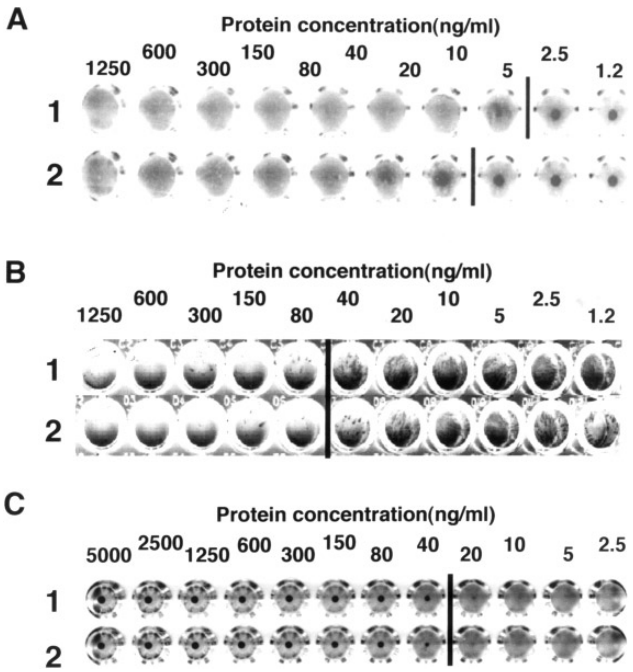

FIG. 3. Comparison of biological activities of recombinant and native conglutinin. (A) Conglutination, (B) inhibition of haemagglutination of influenza A virus, (C) neutralization of influenza A virus. Lane 1, recombinant conglutinin; lane B, native conglutinin.

of full-length C-type lectins with a collagenous domain. The complexity of assembly and post-translational modifications of these proteins, such as glycosylation and proline hydroxylation, has generally required the utilization of eukaryotic expression systems. Initially, the baculovirus-insect cell system was thought to be the most effective method for producing large amounts of recombinant proteins. Surfactant protein-A (SP-A), a bouquet form of lung collectin expressed in baculovirus, has been shown to be abnormally assembled, to lack hydroxyproline, and to contain abnormal N-linked oligosaccharides (21). This suggested that finding a mammalian expression system was essential for obtaining collectins with favorable biochemical characteristics. SP-A cDNAs and genomic sequences have been expressed in CHO (22) and COS cells (23), and the molecules produced show evidence of prolyl hydroxylation, N-linked glycosylation, trimer formation, as well as the expected biological activities in certain functional assays. The recombinant forms of human MBP, a bouquet form of serum collectin, have also been expressed and found to be biologically active. Rat SP-D, a lung cruciform collectin, has been expressed in CHO cells with the correct assembly and post-translational modifications. However, there is nothing in the literature describing the expression of serum cruciform collectins.

In this report, we have described the expression of conglutinin, a serum type of cruciform collectin in a mammalian cell. The recombinant conglutinin migrated in a manner similar to that of native conglutinin on reduced and non-reduced SDS-PAGE, as well as on native PAGE. These results suggested that the recombinant conglutinin underwent proper assembly. Amino acid analysis of native and recombinant conglutinin produced with or without vitamin C showed that further post-translational hydroxylation of proline and lysine was dependent on the presence of vitamin C. The recombinant conglutinin produced in the presence and absence of vitamin C exhibited almost identical inhibition kinetics with N-acetyl-D-glucosamine. The binding

TABLE 2

Sugar Specificity of Native Conglutinin, Recombinant Conglutinin Produced in This Study and Recombinant Conglutinin Carbohydrate Recognition Domain Expressed in *E. coli*

	I_{50} (mM)[a]		
Sugar	Native BKg	recBKg	recBKg-CRD
GlcNAc	1.4	2.5	0.65
Man	19.5	25	12.3
Glc	41.5	48	39.5
Gal	>100	>100	46.8
Mal	49	42	25.8

Note. Abbreviations: BKg, bovine conglutinin; BKg-CRD, bovine conglutinin composed of carbohydrate recognition domain; GlcNAc, N-Acethyl-D-glucosamine; Man, mannose; Glc, glucose; Gal, galactose; Mal, maltose.

[a] The value shows the concentration of sugars required for 50% inhibition of binding. The value for native and recombinant conglutinin CRD were taken from (16) and (17), respectively.

activity of our recombinant conglutinin to the five sugars employed was similar to that of native conglutinin. These results indicated that the fundamental hydroxylation of proline and lysine in the absence of vitamin C, was enough to exhibit the biochemical activity and furthermore hydroxylation in the presence of vitamin C added the stability of structure by increasing the oligomeric form.

The recombinant conglutinin expressed in *E. coli* exhibited a binding specificity to sugars which was similar to that of native conglutinin. It also inhibited hemagglutination by influenza A virus, even though it had 20 times less conglutination activity than the native form (14) and no ability to neutralize the virus (unpublished data). The recombinant conglutinin expressed here showed several biological activities in common with the native conglutinin. These results suggest that the entire conglutinin structure is essential for display of the full activity of this molecule.

In summary, a functional conglutinin was successfully expressed in CHO DG44 cells. This system should provide a useful model for studying conglutinin synthesis and secretion and for further structural and functional studies employing molecular mutagenesis. In addition, this conglutinin will be a useful tool for studies on the immunological role of conglutinin in vivo.

ACKNOWLEDGMENTS

We are grateful to Professor Takachika Azuma for useful discussions and encouragement. We thank Dr. Junichi Kajiwara for amino acid analysis of conglutinin proteins and Dr. William Campbell for critically reading the manuscript. This work was partially supported by the Fuso Pharmaceutical Industry and Sankyo Foundation of Life Science.

REFERENCES

1. Bordet, J., and Gay, F. P. (1906) *Ann. Inst. Pasteur* **20,** 467–498.
2. Bordet, J., and Streng, O. (1909) *Zentralbl. Bankteriol. Parasitenk. Infectionskr. Hyg. Abt I Orig.* **49,** 260–276.
3. Malhotra, R., Haurum, I., Thiel, S., and Sim, R. B. (1992) *J. Immunol.* **22,** 1437–1445.
4. Lachmann, P. J. (1967) *Adv. Immunol.* **6,** 479–521.
5. Hirani, S., Lambris, J. D., and Muller-Eberhard, H. J. (1985) *J. Immunol.* **134,** 1105–1109.
6. Leon, M. A. (1957) *Proc. Soc. Exp. Biol. Med.* **96,** 202–204.
7. Leon, M. A., and Yokohari, R. (1964) *Science* **143,** 1327–1328.
8. Super, M., Thiel, S., Lu, J., Levinsky, R. J., and Turner, M. W. (1989) *Lancet* **2,** 1236–1239.
9. Wakamiya, N., Okuno, Y., Sasao, F., Ueda, S., Yoshimatsu, K., Naiki, M., and Kurimura, T. (1992) *Biochem. Biophys. Res. Comm.* **187,** 1270–1278.
10. Hartshorn, K. L., Sastry, K., Brown, D., White, M. R., Okarma, T. B., Lee, Y. M., and Tauber, A. I. (1993) *J. Immunol.* **151,** 6265–6273.
11. Suzuki, Y., Yin, Y., Makino, M., Kurimura, T., and Wakamiya, N. (1993) *Biochem. Biophys. Res. Commun.* **191,** 335–342.
12. Liou, L. S., Sastry, R., Hartshorn, K. L., Lee, Y. M., Okarma, T. B., Tauber, A. I., and Sastry, K. N. (1994) *Gene* **141,** 277–281.
13. Lu, J., Laursen, S. B., Thiel, S., Jensenius, J. C., and Reid, K. B. (1993) *Biochem. J.* **292,** 157–162.
14. Eda, S., Suzuki, Y., Kase, T., Kawai, T., Ohtani, K., Sakamoto, T., Kurimura, T., and Wakamiya, N. (1996) *Biochem. J.* **316,** 43–48.
15. Lim, B. L., and Holmskov, U. (1996) *Biochem. Biophys. Res. Commun.* **218,** 260–266.
16. Wang, J. Y., Kishore, U., and Reid, K. B. (1995) *FEBS Lett.* **376,** 6–10.
17. Haurum, J. S., Thiel, S., Haagsman, H. P., Laursen, S. B., Larsen, B., and Jensenius, J. C. (1993) *Biochem. J.* **293,** 873–878.
18. Okuno, Y., Tanaka, K., Baba, K., Maeda, A., Kunita, N., and Ueda, S. (1990) *J. Clin. Microbiol.* **28,** 1308–1313.
19. Kawasaki, N., Yokota, Y., and Kawasaki, T. (1993) *Arch. Biochem. Biophys.* **305,** 533–540.
20. Lu, J., Wiedemann, H., Holmskov, U., Thiel, S., Timpl, R., and Reid, K. B. M. (1993) *Eur. J. Biochem.* **215,** 783–799.
21. Kalina, M., McCormack, F. X., Crowley, H., Voelker, D. R., and Mason, R. J. (1993) *J. Histochem. Cytochem.* **41,** 57–70.
22. McCormack, F. X., Fisher, J. H., Suwabe, A., Smith, D. L., Shannon, J. M., and Voelker, D. R. (1990) *Biochem. Biophys. Acta* **1087,** 190–198.
23. Alcorn, J. L., Chen, Q., Boggaram, V., and Mendelson, C. R. (1992) *Am. J. Physiol.* **262,** L437–L445.