BB2-crystallin, the major component of β-crystallin, is a dimer at low concentrations but can form oligomers under physiological conditions. The interaction domains have been speculated to be the β-sheets, each of which is formed by two or more β-strands. BB2-crystallin consists of 16 β-strands, 8 in the N-terminal domain and 8 in the C-terminal domain. Domain interaction sites may be predicted from the PHD method (6). The distribution for conditions. The interaction domains have been speculated to be the low concentrations but can form oligomers under physiological hybrid system assay. Protein-protein interactions decreased for all protein-protein interactions was screened by a mammalian two-hybrid system assay. Protein-protein interactions decreased for all β-strand-deleted mutants except 120E, L34E, and L162E mutants; this effect was not seen in the two mutant controls, V46E and V129E. The sequences around Val-54, Val-60, Val-73, and Leu-97 in the C-terminal region removes three β-strands that participate in intermolecular interactions. Some selected mutant proteins that showed strong (V46E and V129E) and reduced (V60E, V144E, V60N, and V144N) interactions were expressed in bacterial culture and were studied with spectroscopy and chromatography. The V60E and V144E mutants were found to be partially unfolded and incapable of forming a complete dimer.

BB2-crystallin is the major component of β-crystallin and is a dimer at low concentrations (1–3). At high concentrations or in the lens, BB2-crystallin forms hetero-oligomers with other β-crystallins. These oligomeric β-crystallins further participate in the formation of a supramolecular assembly that is important in lens function-lens transparency. A molecular assembly that is important in lens function-lens transparency. A molecular assembly that is important in lens function-lens transparency.

MATERIALS AND METHODS
Subcloning Mutant Genes into the Two-hybrid System Vectors—We used the Clontech Mammalian Two-Hybrid System Assay Kit 2 (Clontech, Palo Alto, CA). The test protein (bait) was fused into the GAL4 DNA-BD in the pM vector and the second test protein (prey) was fused into the VP16-AD in the pVP16 vector. The third vector, pG5SEAP, contains a reporter construct and is encoded with secreted alkaline phosphatase (SEAP), an enzyme that enables sampling of culture medium without cell lysis.

We used the previously constructed plasmids pM-βB2 and pVP16-βB2 (11, 12) to prepare 14 mutants of X → Glu substitution using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The primers used were custom synthesized (Invitrogen) (Table 2). The corresponding mutants of X → Asn substitution were also prepared.

Cotransfection and SEAP Assay—HeLa cells were used in the cell culture, and Lipofectamine 2000 (Invitrogen) was used in cotransfection as previously reported (10–12). HeLa cells were grown at 37 °C with 5% CO2 with 10% serum and were seeded at 2 × 105 cells in 500 µl of medium/well in a 24-well plate. Plasmids pM-X (0.3 µg) and pVP16-Y (0.3 µg) and pG5SEAP reporter vector (0.3 µg) were added to the wells containing 2 µl of Lipofectamine 2000 reagent.

SEAP activity was detected 48 h after transfection using the BD Great EscAPE SEAP fluorescence detection kit (Clontech). The detailed protocol for SEAP detection is provided in the kit and was used in our recently reported study (10). The fluorescence substrate MUP
Mutants of B2-crystallin were prepared by substituting GAG or GAA with AAC in the forward primers and CTC or TTC with GTT.

**TABLE 1**

| N-terminal extension | 1 | 11 |
|----------------------|---|----|
| βB2<sup>a</sup> | MASDHQTQAG | KPSLNL |
| βB2<sup>b</sup> | | LN |

**Motif I**

| βB2<sup>a</sup> | 17 | 27 |
|------------------|----|----|
| PKIIIFEGEN | FQGHSHELNG |
| PKIIIFEGEN | FQGHSHELNG |
| GITTEYEDRG | FQGHCXCSS |

| βB2<sup>b</sup> | 37 | 47 |
|------------------|----|----|
| GPPVGYEQAN | EKAGSVLQVA |
| GPPVGYEQAN | EKAGSVLQVA |
| GCWMLYERPN | SRCNSIRVDS |

| βB2<sup>a</sup> | 57 | 67 |
|------------------|----|----|
| GPWVGYQAN | GEPRWDSWT |
| GPWVGYQAN | GEPRWDSWT |
| GCWMLYERPN | GDYDPQYQWW |

| βB2<sup>b</sup> | 77 | 87 |
|------------------|----|----|
| CCCGGCTACCGTG | SRRRTSDSS |
| CCCGGCTACCGTG | SRRRTSDSS |
| TGGCTACG | G-F-NDSSS |

**Motif II**

| βB2<sup>a</sup> | 107 | 117 |
|------------------|-----|-----|
| HKIIYLEPN | FTGK<sub>107</sub>QID |
| HKITLYENPN | FTKKKMEVID |
| FRMRIYERDD | FROGQSMIT- |

| βB2<sup>b</sup> | 127 | 137 |
|------------------|-----|-----|
| GGGTACTGGCAGTT | DDPSFHAHAG |
| GGGTACTGGCAGTT | DDPSFHAHAG |
| GGGTACTGGCAGTT | DDPSLQDQR |

| βB2<sup>a</sup> | 147 | 157 |
|------------------|-----|-----|
| YOEKVSVRF | QSYSCQSRV |
| YOEKVSVRF | QSYSCQSRV |
| HLTVEHSLNV | LE |

**Motif III**

| βB2<sup>a</sup> | 149 | 159 |
|------------------|-----|-----|
| GTWVGYQYPG | YRGQLYLELEK |
| GTWVGYQYPG | YRGQLYLELEK |
| GSYWLYEMSP | YRGROYYRLR |

| βB2<sup>b</sup> | 169 | 179 |
|------------------|-----|-----|
| GGGTACATCGTC | GDDYKSSDFG |
| GGGTACATCGTC | GDDYKSSDFG |
| GGGTACATCGTC | GEVRYLWDWG |

**Motif IV**

| βB2<sup>a</sup> | 197 | 207 |
|------------------|-----|-----|
| GGGGTTTTCAC | APHPQVQSRV |
| GGGGTTTTCAC | APHPQVQSRV |
| GGGGTTTTCAC | AMNAVQSGL |

| βB2<sup>b</sup> | 217 | 227 |
|------------------|-----|-----|
| GGGGTTTTCAC | RIR |
| GGGGTTTTCAC | RIR |
| GGGGTTTTCAC | RVMDFY |

**C-terminal extension**

| βB2<sup>a</sup> | 192 | 202 |
|------------------|-----|-----|
| DMQWHQQRGAF | HPSN |

| βB2<sup>b</sup> | 202 | 212 |
|------------------|-----|-----|
| DMQWHQQRGAF | HPSN |

<sup>a</sup>The prediction from PHD program by Rost and Sander (6) for the wild-type human lens β2-crystallin. The sequence numbering is based on the sequence of the wild-type β2-crystallin. The sequences of the β-strands are shown in bold.

<sup>b</sup>-The distribution of β-strand is taken from Box et al. (5) for bovine lens β2-crystallin and Kumaraswamy et al. (7) for bovine lens yB-crystallin.

**TABLE 2**

The forward and reverse primers for subcloning β2-crystallin mutants of X → Glu substitution

The primers for the corresponding mutants of X → Asn substitution were prepared by substituting GAG or GAA with AAC in the forward primers and CTC or TTC with GTT.

| βB2<sup>a</sup> | 2625 |
|------------------|-----|
| L20E | Forward: GTCCCTCAGCCCAAGATAGQTACGTTTGGAAGAGGA |
| L20E | Reverse: CCTCCCTCAGCCCAAGATAGQTACGTTTGGAAGAGGA |

| βB2<sup>b</sup> | 2625 |
|------------------|-----|
| L34E | Forward: GGCCTCTCCAGCCCAAGATAGQTACGTTTGGAAGAGGA |
| L34E | Reverse: GGCCTCTCCAGCCCAAGATAGQTACGTTTGGAAGAGGA |

**Expression of βB2-Crystallin Mutants**—The use of the QIAexpression Type IV kit (Qiagen, Valencia, CA) in the cloning, expression, and purification of His<sub>6</sub>-tagged β2-crystallin has been described elsewhere (10). Briefly, the β2-crystallin mutant genes in plasmids (e.g. pM-βB2WT) were amplified by PCR using pETu DNA polymerase (Stratagene) with the forward/reverse primers: CCGGTATACCCGGCTCATCACATCCAG/CCCAGTGGGTGGTTGGAAGGTTGAA. Two restriction sites, KpnI and HindIII, were included in the primers. The PCR products and pQE-30 vector were doubly digested by KpnI and HindIII. The digested genes and vector were then ligated by DNA ligase under standard conditions. The β2 cDNA inserts were verified by sequencing analysis.

The expression constructs containing various β2-crystallin genes were transformed into Escherichia coli strain M15 (pREP4). Cell culture was performed to induce expression of various proteins by a standard protocol. The His<sub>6</sub>-tagged β2-crystallins, either wild-type or mutant, were purified by nickel-nitrilotriacetic acid affinity chromatography.

**SDS-PAGE and Immunoblotting**—SDS-PAGE was performed in a slab gel (15% acrylamide) under reducing conditions according to the method of Laemmli (13). Western blotting was performed with polyclonal anti-β-crystallin antibodies (a gift from Sam Zigler of NEI/National Institutes of Health). Protein concentrations were determined by (4-methylumbelliferyl phosphate) provides an easy assay of SEAP by reading fluorescence at 360/449 nm. A standard linear curve was obtained using the positive placentual alkaline phosphatase. The expression of GAL4 DNA-BD/protein fusion was verified by Western blot with the GAL4 DNA-BD monoclonal antibody (11, 12). To see whether expression levels were a factor for different SEAP activities, protein concentrations in the soluble protein extracts were determined using the Pierce BCA assay. The results indicated that protein expression levels varied very little among the various culture cells.
measuring absorption at 280 nm: $A^{0.1%} = 1.74$ for both WT and mutant βB2-crystallins (14).

**FPLC Gel Filtration**—Size-exclusion chromatography was carried out in fast protein liquid chromatography (FPLC) with a Superose-12 column.

**Spectroscopic Studies**—CD spectra were obtained with an Aviv circular dichroism spectrometer (model 60 DS; Aviv Associates, Lakewood, NJ). Five scans were recorded and averaged and followed by a polynomial-fitting program. The CD was expressed as deg-cm$^2$-dmol$^{-1}$.

Fluorescence was measured with a Shimadzu spectrofluorometer (model RF-5301PC; Shimadzu Instruments, Columbia, MD). Trp emission was scanned with an excitation wavelength at 295 nm. Bis-ANS fluorescence emission spectra were scanned between 460 and 560 nm with an excitation wavelength of 395 nm.

**RESULTS**

**Protein-Protein Interactions Involving βB2-Crystallin Mutants**—The SEAP activities as detected by 4-methylumbelliferyl phosphate fluorescence between βB2-crystallin mutants themselves are shown in Fig. 1. The majority of the mutations in the β-strands, including V54E, V60E, V73E, L97E, L109E, L124E, V144E, V152E, L165E, and V187E, decreased SEAP activities relative to the wild-type βB2-crystallin. The mutations that did not decrease SEAP activities included I20E, L34E, V46E, V129E, and L162E. In general, mutations in the C-terminal region reduced SEAP activity more than did those in the N-terminal region. The two control mutations, V46E and V129E, which are not in the β-strand, did not decrease the SEAP activity. These results indicated that disruption of particular β-strands that might participate in dimerization was responsible for the reduction of SEAP activity. Those mutations that resulted in low SEAP activities between themselves (e.g. V54E-V54E) also showed low SEAP activities with WT (e.g. V54E-WT) (Fig. 2).

**Biophysical Studies of βB2-Crystallin Mutants**—Four βB2-crystallin mutants with a Val→Asn substitution, V60N and V144N mutants were prepared. SDS-PAGE, Western Blot, and FPLC Size-exclusion Chromatography—SDS-PAGE and Western blot are shown in Fig. 4. The His$_6$-tagged WT βB2-crystallin did not show any difference in Trp fluorescence and CD from non-His$_6$-tagged WT βB2-crystallin (10). For mutation of Val→Asn substitution, V60N and V144N mutants were prepared.

**SDS-PAGE, Western Blot, and FPLC Size-exclusion Chromatography**—SDS-PAGE and Western blot are shown in Fig. 4. The His$_6$-tagged βB2-crystallin genes were fused into the pM containing the DNA binding domain (BD) and a pVP16 vector containing the transcript activity domain (AD) and cotransfected to HeLa cells with pG5SEAP reporter vector. The culture medium was assayed for SEAP activity. Statistical significance was calculated by the paired t-test. Significant decreases were observed for the mutants from WT (*, $p < 0.05$; **, $p < 0.005$).

**RESULTS**

**Protein-Protein Interactions Involving βB2-Crystallin Mutants**—The SEAP activities as detected by 4-methylumbelliferyl phosphate fluorescence between βB2-crystallin mutants themselves are shown in Fig. 1.
crystallin is located at a higher molecular weight than the non-tagged B2-crystallin.

The elution profiles of the V46E and V129E mutants on FPLC are the same as that of the WT, but those of V60E and V144E mutants appeared to contain a mixture of dimers and monomers (Fig. 5). A rerun of collected fractions between 18 and 21 ml for V144E mutant showed the same profile. The corresponding mutants V60N and V144N did not show the effect.

CD and Fluorescence—Both far-UV and near-UV CD changed greatly for the V60E and V144E mutants (Figs. 6 and 7), indicating an alteration occurs not only in the secondary structure but also the tertiary structure. The change is similar for the two mutants, but the other two control mutants, V46E and V129E, did not show these changes. The CD data indicate that the mutations (Val → Glu) involving the addition of charge change CD more than those mutations (Val → Asn) involving no change of charge.

Trp and Bis-ANS Fluorescence—Trp fluorescence showed no shift in emission maximal wavelength and only a small change in intensity for V60E and V129E mutants but a large red shift (from 331 to 345 nm) for V60E and V144E or V60N and V144N mutants (Fig. 8), suggesting that Trp residues in these mutants are in a more hydrophilic environment than are those in V46E, V129E, and WT.

In comparison with αA-crystallin, WT Bβ2-crystallin gave a relatively low bis-ANS fluorescence intensity (2), but the intensity increased greatly for the V60E and V144E or V60N and V144N mutants (Fig. 9), indicating that the buried hydrophobic surfaces became accessible to the bis-ANS probe in these mutants.
The crystallographic structure of βB2-crystallin has been determined for a homodimer of a fragment of a 181-amino acid sequence without the N- and C-terminal extensions (5, 15) and a tetrameric WT (16). The distribution of the β-strand in each subunit is different in dimers and tetramers. For the purpose of our study, the use of the crystallographic structure of homodimer βB2-crystallin should be adequate. Both the dimer and tetramer structures were determined with bovine lens βB2-crystallin. Because we used human lens βB2-crystallin, we used a PHD program to predict the secondary structure (6). The predicted secondary structure is in good agreement with that from crystallography (Table 1). It is reasonable to assume that the structure of human βB2-crystallin is similar to that of bovine βB2-crystallin, because they share a high sequence homology (91%). We also included the distribution of β-strands for γB-crystallin for comparison because the structures of βB2- and γB-crystallin are similar. Both βB2- and γB-crystallin structures are characterized by having four Greek key motifs: two (1 and 2) in the N-terminal domain and two (3 and 4) in the C-terminal domain (5, 7). Each motif consists of four β-strands, and four motifs form four β-sheets. Each β-sheet contains four β-strands that do not correspond exactly to those in the particular motif. The β1-sheet consists of three β-strands in motif-1 (β1-, β2-, and β3-) and one β-strand in motif-2 (β4-). The corresponding β-strands in the other three β-sheets are: β2-sheet (β2-, β3-, β4-, and β5-), β4-sheet (β5-, β6-, β7-, and β8-), and β3-sheet (β6-, β7-, β8-, and β9-). In γB-crystallin, the two domains are connected by a flexible linker peptide and organized so that two β-sheets (sheet-1 and sheet-3) lie on the outside of the molecule and the other two (sheet-2 and sheet-4) are in partial contact (intramolecular domain association). βB2-crystallin has a structure similar to that of γ-crystallin except that the linker peptide is extended in a way favoring an intermolecular association; the two subunits associate in a dimer in a swap form such that the N-terminal domain of the first subunit is adjacent to the C-terminal domain of the second subunit. The other major
difference is that βB2-crystallin contains an N-extension and a C-extension, reported to be responsible for preventing oligomerization (association of dimers) (17, 18). Domain interactions in the tension, reported to be responsible for preventing oligomerization/H9252 important role in dimerization. However, the results of some mutations first subunit, and the sheet-4 consists of the motif-2, and the mutations in Val-152, Leu-165, and Val-187 destroyed strand in the C-terminal region of the second subunit. The mutations in H9252 concentration is 0.08 mg/ml. The excitation wavelength is 295 nm: Val H9252 not in the H9252 decrease SEAP activities. The mutation sites of Val-46 and Val-129 are H9252 of the second subunit was not entirely responsible for the dimerization. H9252 of the second subunit was not entirely responsible for the dimerization. The Val → Glu substitution introduced a negative charge: we made some Val → Asn substitutions to see whether the decrease of SEAP activity might be due to the introduction of the charge and found that the changes in SEAP activities were basically the same as that of mutants with the X → Glu substitution. The disruption of key β-strands is the critical mechanism for the decrease of SEAP activity. The observation that V60E (V60N) and V144E (V144N) mutations greatly reduced SEAP activity indicated that the β5- and β12-strands were two of the many dimerization sites, which was further confirmed by their inability to form complete dimers. The control mutants V46E and V129E, in which mutations were in the non-β-strand, did not show these effects. The partial unfolding in the V144E mutant was also seen in the V144N mutant, indicating that disruption of the β-strand by either a Val → Glu or a Val → Asn substitution gave the same result.

As dimerization is the initial step in the formation of β-crystallin complexes seen in the human lens, to maintain the integrity of the subunit interaction domains is thus important in sustaining their stability. Any disruption of the domain structures by either posttranslational modification or mutation will disturb the complex formation that is critical for lens transparency. This was demonstrated in the congenital cataract CRYBB2 gene product Q155*, in which 51 amino acid residues extending the present study to βB2-crystallin but also for αA- and αB-crystallin, whose three-dimensional structures have not been established. It is possible to obtain data about dimerization domains similar to those of βB2-crystallin by extending the present study to α-crystallin, with its β-strand distribution predicted by the PHD program. The sites of both R116C αA-crystallin and R120G αB-crystallin mutations, associated with various autosomal dominant congenital cataracts, are located in the β7-strand and were found to change protein-protein interactions profoundly (11),...
although the mechanism might not be related to disruption of the β-strand. Two recent studies reported the interaction domains for αB-crystallin, with their results not in complete agreement; one used a peptide scan (19), and the other used a protein pin array (20). The latter report indicates that interaction domains are accessible to solvent and consist primarily of β-strands in the core region with a minor helical structure in the N-terminal region and a nonstructural sequence in the C-terminal region. An earlier study using site-directed spin labeling identified an interface consisting of two β-sheets of seven β-strands at the α-crystallin domain that mediates the formation of dimeric αA-crystallin (21). The other relevant study was reported by J. King and coworkers (22) on human γD-crystallin. Using site-specific mutagenesis on γD-crystallin and an unfolding-refolding study, they found that some hydrophobic residues (Phe-56, Ile-81, Val-132, and Leu-145) are important in the domain interactions and they are in the β-strands that contribute to intra-domain interactions.

In summary, we have demonstrated that the two-hybrid system could be used to determine the subunit interaction domains for βB2-crystallin. Our studies confirm the key β-strands that play an important role in the dimerization.

REFERENCES
1. Lampi, K. J., Ma, Z., Shih, M., Shearer, T. R., Smith, J. B., Smith, D. L., and David, L. L. (1997) J. Biol. Chem. 272, 2268-2275
2. Fu, L., and Liang, J. J. (2001) Mol. Vis. 7, 178-183
3. Hejtmancik, J. F., Wingfield, P. T., and Sergeev, Y. V. (2004) Exp. Eye Res. 79, 377-383
4. Driessen, H. P., Bax, B., Slingsby, C., Lindley, P. F., Mahadevan, D., Moss, D. S., and Tickle, I. J. (1991) Acta Crystallogr. Sect. B Struct. Crystallogr. 47, Pt. 6, 987-997
5. Bax, B., Lapatto, R., Nalini, V., Driessen, H., Lindley, P. F., Mahadevan, D., Blundell, T. L., and Slingsby, C. (1990) Nature 347, 776-780
6. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584-599
7. Kumaraswamy, V. S., Lindley, P. F., Slingsby, C., and Glover, I. D. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. 52, 611-622
8. Chou, P. Y., and Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251-276
9. Chou, P. (1989) in Prediction of Protein Structure and the Principles of Protein Conformation (Fasman, G. D., ed) pp. 549-586, Plenum Press, New York
10. Liu, B. F., and Liang, J. J. (2005) Mol. Vis. 11, 321-327
11. Fu, L., and Liang, J. J. (2003) Invest. Ophthalmol. Vis. Sci. 44, 1155-1159
12. Fu, L., and Liang, J. J. (2002) J. Biol. Chem. 277, 4255-4260
13. Laemmli, U. K. (1970) Nature 227, 680-685
14. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) Anal. Biochem. 200, 74-80
15. Bax, B., and Slingsby, C. (1989) J. Mol. Biol. 208, 715-717
16. Nalini, V., Bax, B., Driessen, H., Moss, D. S., Lindley, P. F., and Slingsby, C. (1994) J. Mol. Biol. 236, 1250-1258
17. Trinkl, S., Glockshuber, R., and Jaenicke, R. (1994) Protein Sci. 3, 1392-1400
18. Sergeev, Y. V., Hejtmancik, J. F., and Wingfield, P. T. (2004) Biochemistry 43, 415-424
19. Sreelakshmi, Y., Santhoshkumar, P., Bhattacharyya, J., and Sharma, K. K. (2004) Biochemistry 43, 15785-15795
20. Ghosh, J. G., and Clark, J. I. (2005) Protein Sci. 14, 684-695
21. Koteiche, H. A., and McHaourab, H. S. (1999) J. Mol. Biol. 294, 561-577
22. Kosinski-Collins, M. S., Flaugh, S. L., and King, J. (2004) Protein Sci. 13, 2223-2235