Concoxin (Cx) 45.6, an avian counterpart of rodent Cx50, is phosphorylated in vivo, but the sites and function of the phosphorylation have not been elucidated. Our peptide mapping experiments showed that the Ser363 site in the carboxyl (COOH) terminus of Cx45.6 was phosphorylated and that this site is within casein kinase (CKII) II consensus sequence, although showing some similarity to CKII sequence. The peptide containing Ser363 could be phosphorylated in vitro by CKII, but not by CKI. Furthermore, CKII phosphorylated Cx45.6 in embryonic lens membrane and the fusion protein containing the COOH terminus of Cx45.6. Two-dimensional peptide mapping experiments showed that one of the Cx45.6 peptides phosphorylated in vivo migrated to the same spot as one of those phosphorylated by CKII in vitro. Furthermore, CKII activity could be detected in lens lysates. To assess the function of this phosphorylation event, exogenous wild type and mutant Cx45.6 (Ser363→Ala) were expressed in lens primary cultures by retroviral infection. The mutant Cx45.6 was shown to be more stable having a longer half-life compared with wild type Cx45.6. Together, the evidence suggests that CKII is likely a kinase responsible for the Ser363 phosphorylation, leading to the destabilization and degradation of Cx45.6. The connexin degradation induced by phosphorylation has a broad functional significance in the regulation of gap junctions in vivo.

Gap junctions are channels between two adjacent cells, which allow passage of small molecules ($M_r \leq 1000$) such as small metabolites, ions, and second messengers. The gap junction-mediated cell-to-cell communications are important in maintaining cell and tissue functions (1). The structural components of gap junctions are members of a family of related membrane proteins called connexins, which consist of four conserved transmembrane domains and two extracellular loops, while their cytoplasmic regions are unique. The COOH terminus, the most variable region among connexins, contains several putative kinase consensus sequences. The phosphorylation of connexins has been indicated in the intracellular trafficking of connexins (2), channel assembly (3), gating of gap junctional communication (4), stabilization of connexins from degradation (5, 6), etc. In isolated myocardial gap junctions, most Cx43 phosphorylation sites are in the region 271–382 (7).

The vertebrate eye lens is one of the important model systems used in the study of the function and regulation of gap junctions. The lens is an avascular organ formed by an anterior epithelial cell layer and highly differentiated fiber cells. To maintain their metabolic activities and homeostasis, the cells inside the lens must depend fully on extensive networks of gap junction-mediated cell-to-cell communications with the cells on the lens surfaces (8), although the report by Bassnett et al. (9) shows the lack of gap junction-mediated transport from the lens epithelium to the fibers. Primary cultures of chick lens closely mimic in vivo lens cell differentiation processes in which monolayer lens epithelial cells differentiate into structures called “lentoids” having features of differentiated lens fibers (10, 11). Lens fiber connexins in the chick primary cultures are phosphorylated similarly to the in vivo phosphorylated connexins (12, 13), while those in the primary cultures of rodent and bovine lenses are not processed into proper phosphorylated forms (14). There are three connexins identified in the chick lens: Cx43, which is expressed in anterior lens epithelial cells and in the lens bow region (15) and Cx45.6 and Cx56, which are present mainly in lens fiber cells (16, 17) and to a lesser degree in junctions between epithelial cells (17, 18). All three connexins are phosphoproteins (16, 19, 20). PKC1 is one of the kinases that may be involved in the phosphorylation of lens fiber connexins (12, 13). Two potential phosphorylation sites of Cx56 stimulated by PKC activator, 12-O-tetradecanoylphorbol-13-acetate have been localized at the intercellular loop and COOH-terminal regions (13).

Besides other potential roles of connexin phosphorylation, one functional feature of phosphorylation involves either stabilization or destabilization of various connexins in different systems. Phosphorylation-induced degradation of Cx43 has been observed in rat liver epithelial cells following 12-O-tetradecanoylphorbol-13-acetate treatment (21) and in mouse keratinocytes following hepatocyte growth factor/scatter factor treatment (22). Stimulation of Cx43 phosphorylation by platelet-derived growth factor correlates with a faster turnover of Cx43 utilizing the lysosomal pathway (23). In contrast, for mouse Cx45 it has been shown that decreased phosphorylation coincided with degradation of this protein (2). The substitution of one or more serine residues for other amino acids increases degradation of the transfected Cx45 in HeLa cells (5). Since in vivo phosphorylation sites for any of the above mentioned connexins have not been mapped, the direct relationship between in vivo phosphorylation and connexin degradation is not clear. In the vertebrate lenses, the phosphorylation of chick fiber Cx56 is apparently involved in the stability of the protein (24).

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¶ The abbreviations used are: PKC, protein kinase C; Cx, connexin; CKI, casein kinase I; CKII, casein kinase II; GST, glutathione S-transferase; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; TPCK, Tos-PheCH₂Cl; MOPS, 4-morpholinepropanesulfonic acid.
The phosphorylation level of lens fiber connexins increases and is associated with lens development (14), but the exact nature of this increase has not been elucidated. In the center of the lens referred to as the lens nucleus, the more differentiated region, sheep lens Cx49, the ovine homologue of Cx45.6 is cleaved at the COOH-terminal region by calpain, a Ca\(^{2+}\)-dependent protease (25).

Although mechanisms and functions of connexin phosphorylation have been studied extensively, the exact roles of in vivo phosphorylation, particularly the direct correlation to a specific in vivo phosphorylation site to its function, are less certain. To the extent of our knowledge, this is the case for all connexins. In this report, we show directly by peptide mapping and tandem mass spectrometric (MS) sequencing that Cx45.6 is phosphorylated in vivo at Ser\(^{363}\) in the COOH-terminal domain. Ser\(^{363}\) is localized within CKII phosphorylation consensus sequence (26). CKII is a cyclic nucleotide- and calcium-independent serine/threonine protein kinase (26). Our sequence (26) is phosphorylated by CKII to explore the functional significance of Ser\(^{363}\) phosphorylation, we expressed exogenous wild type Cx45.6 and mutant Cx45.6 (Ser\(^{363}\) → Ala) into chick lens primary cell cultures using a retroviral approach. Pulse-chase experiments revealed that the mutant Cx45.6 lacking Ser\(^{363}\) had an extended half-life compared with the wild type Cx45.6. The results suggest that phosphorylation of Ser\(^{363}\) by CKII may provide signal(s) for Cx45.6 destabilization and degradation, which may lead to the in vivo regulation of gap junctions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fertilized chicken eggs were obtained from SPAFAS (Ronkonkoma, IL) and Tyson Hatchery (Gonzalez, TX). CKII, heparin, and CKI consensus peptide (RRDLHIDEDEEMSAITA) were obtained from Sigma. CKI was purchased from Promega (Madison, WI). The peptide derived from Cx45.6 (RRREEEVEYVS\(^{362}\)DEVE\(^{367}\)) and CKII consensus peptide (RRREEEVEYVS\(^{362}\)DEVE\(^{367}\)) were synthesized in the Institutional Peptide Laboratory, PKC peptide, (CGTPEYLAPEIRR) was kindly provided by Dr. F. Liu at the University of Texas Health Science Center at San Antonio. Whatman P81 phosphocellulose paper was from Fisher Scientific (Pittsburgh, PA). 32P-labeled and labeled proteins for mass spectrometric (MS) sequencing that Cx45.6 is phosphorylated in vivo at Ser\(^{363}\) in the COOH-terminal domain.

**Mapping and Sequencing of the Cx45.6 Phosphorylation Sites by Nanoelectrospray Mass Spectrometry**—SDS-PAGE: The labeled bands were identified by autoradiography and excised from dried gels. The gel slices were washed with 32% ethanol and 42% acetic acid for 30 min and incubated at 37 °C for 1 h. The gel slices were dried completely in speed vacuum and rehydrated into 200 μl of TBO-treated trypsin (10 mg/ml) and 50 mM NH\(_4\)HCO\(_3\) (pH 8.0) at 37 °C for 1 h, followed by the addition of 100 μl of trypsin every 20 min three times and incubated for 16 h. The supernatant was transferred, dried, and washed several times with H\(_2\)O. The final tryptic digested products were resuspended in electrophoresis buffer and separated on two dimensions. 

**Connexin Phosphorylation by Casein Kinase II**

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**In Vivo Phosphorylation of the Synthetic Peptide by CKII/CKI**—The experimental procedures for peptide phosphorylation by CKII/CKI were based on the established methods (31, CKI Users Manual, Promega) with some modifications. The peptide phosphorylation was initiated by addition of 0.5 μl of CKII (122 unit/ml) or 1 μl of CKI (10 unit/ml) to 25 μl of CKII or CKI phosphorylation buffer plus 1 μm synthetic peptides. CKII phosphorylation buffer contained 10 mM MgCl\(_2\), 50 mM MOPS (pH 7.0), 150 mM NaCl, and 2.5 μl of [γ-32P]ATP. The substrate peptides in CKII reaction included RRREEEVEYVS\(^{362}\)DEVE\(^{367}\)(Cx45.6, amino acids 358–367) (with or without 80 μg/ml Beparin), RRREEEVEYVS\(^{362}\)DEVE\(^{367}\) (CKII, consensus peptide), or CGTPEYLAPEIRR (PKCα peptide). CKII buffer contained 10 mM MgCl\(_2\), 50 mM Tris-HCl (pH 7.4), 5 mM dithiothreitol, 2.5 μl of [γ-32P]ATP. The substrate peptides in CKII reaction included a peptide of Cx45.6 amino acids 358–367, RRKLHIDEDEEMSAITA (CKI, consensus peptide) or CKII consensus peptide. After incubation at 30 °C for 30 min, the reaction was terminated by 10% trichloroacetic acid (w/v). After incubation at 30 °C (30 min), the gels were washed with acetone once. When dry, the paper was transferred to a scintillation vial to measure radioactivity using a Packard counter (Meriden, CT).

**Preparation of the Fusion Protein, Isolation of the Lens Membrane, In Vitro Phosphorylation by CKII, and Immunoprecipitation**—Fusion proteins containing GST plus the COOH-terminal region of Cx45.6, amino acid 237–400, was produced using the vector pGEX-2T (32). The detailed procedures for purification of fusion protein were described previously (33). The purified GST-Cx45.6 fusion protein and lens membranes were used for phosphorylation reactions. The CKII similar peptide phosphorylation with some modifications: 1 to 5 μg of GST fusion protein or lens membranes were used and the reaction lasted for 2 h. The reaction was terminated by boiling for 5 min in the presence of 0.6% SDS. Immunoprecipitation experiments were performed with in vitro phosphorylated lens membrane samples by Cx45.6 affinity purified antibody as described previously (33).

**Labeling of the Chick Embryonic Lens Organs and Two-Dimensional Tryptic Phosphopeptide Analysis**—Intact lenses were removed from embryonic day 9 chicken as described previously (12) and washed three times with phosphate-free Dulbecco’s modified Eagle’s medium. The lenses were incubated at 37 °C for 16 h in the 32P labeling medium containing phosphate-free Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum and 0.4 μCi/ml H\(^{32}\)PO\(_4\).

Two-dimensional peptide mapping experiments were performed based on modified procedures as described previously (12). Briefly, in vitro phosphorylated GST-Cx45.6 fusion protein and immunoprecipitated samples of 32P-labeled lens organ culture were separated on 10% SDS-PAGE. The labeled bands were identified by autoradiography and excised from dried gels. The gel slices were washed with 32% ethanol and 42% acetic acid for 30 min and incubated at 37 °C for 1 h. The gel slices were dried completely in speed vacuum and rehydrated into 200 μl of TBO-treated trypsin (10 mg/ml) and 50 mM NH\(_4\)HCO\(_3\) (pH 8.0) at 37 °C for 1 h, followed by the addition of 100 μl of trypsin every 20 min three times and incubated for 16 h. The supernatant was transferred, dried, and washed several times with H\(_2\)O. The final tryptic digested products were resuspended in electrophoresis buffer and separated on two dimensions. 

**Preparation of the Phosphorylated Cx45.6 for the Phosphorylation Site Mapping**—Approximately 350 intact lenses were dissected out from 500 embryonic day 18 fertilized chicken eggs. The lenses were rinsed several times in phosphate-buffered saline to free attached cell and tissue debris. The lens membranes were then prepared based on a previously described procedure with some modifications (12, 19). Briefly, lenses were lysed and centrifuged at 100,000 × g (Beckman, 28,000 rpm, SW60TI rotor) for 30 min at 4 °C. The pellets were resuspended in 20 μl of modified trypsin (Promega, Madison, WI) in the presence of aprotinin (100 μg/ml) and incubated at 37 °C for 30 min. The phosphorylation level of lens fiber connexins increases and is associated with lens development (14), but the exact nature of this increase has not been elucidated. In the center of the lens referred to as the lens nucleus, the more differentiated region, sheep lens Cx49, the ovine homologue of Cx45.6 is cleaved at the COOH-terminal region by calpain, a Ca\(^{2+}\)-dependent protease (25).

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Connexin Phosphorylation by Casein Kinase II

RESULTS

Identification of in Vivo Cx45.6 Phosphorylation Site at Ser\(^{363}\) — Prior studies have shown that Cx45.6 is a phosphoprotein (12, 16). To determine the nature of the phosphorylation, we analyzed Cx45.6 by nanoelectrospray MS. Cx45.6 derived from embryonic chick lens was purified by immunoprecipitation followed by SDS-PAGE and the appropriate bands were excised and digested with trypsin in situ. Subsequent nanoelectrospray MS analyses resulted in a tryptic map (Fig. 1A). From this peptide map based on the Cx45.6 sequence, a number of peptides were assigned. Surprisingly, one peptide (see T37–38(Sp)\(^{32P}\) in Fig. 1A) was observed which corresponded to the expected tryptic peptide, T37–38, but was heavier by the mass of one phosphate group (plus 79.4). For this peptide multiple signals (at m/z 1354.5, 903.4 corresponding to doubly and singly charged peptides, respectively) were observed in high resolution scans (data not shown) permitting definite charge and mass assignment.

In order to verify the exact phosphorylation site for this peptide, tandem MS experiments were performed. Fig. 1B shows the tandem MS spectrum for the T37–38(Sp) phosphopeptide. As indicated in this figure, both NH\(_{4}\) terminus-derived fragment ions as well as COOH terminus-derived fragment ions were observed to assign the site of phosphorylation to Ser\(^{363}\). Unlabeled fragment ions correspond to immonium or internal fragment ions. For complete nomenclature of tandem fragment ions, see Roepstorff and Fohlman (48).

Fig. 1. Mass spectrometric analysis of Cx45.6 for in vivo phosphorylation. Nanoelectrospray mass spectrum (A) of Cx45.6 tryptic map from an in-gel digest. Tryptic peptides are numbered sequentially from the NH\(_{2}\) terminus while the superscripts indicate the charge state of each peptide. Trypsin autolysis peptides are indicated by an asterisk.

Tandem mass spectrum (B) of T37–38(Sp)\(^{32P}\) phosphopeptide (m/z 1354.5, K\(^{+}\)AEEEVYSpDEVEGPSAPAELATDVR\(^{380}\)). Sufficient number of complementary B ions (NH\(_{4}\) terminus derived fragment ions) and Y ions (COOH terminus derived fragment ions) were observed to assign the site of phosphorylation to Ser\(^{363}\). Unlabeled fragment ions correspond to immonium or internal fragment ions. For complete nomenclature of tandem fragment ions, see Roepstorff and Fohlman (48).
Phosphorylation of a PeptideCorresponding to Amino Acids 358–367 of Cx45.6 by CKII—Two major features are characteristic of CKI-specific phosphorylation sites: 1) phosphorylated peptides are enriched in Glu and Asp; 2) acidic amino acids are prefered at the positions close to the COOH-terminal side of Ser/Thr rather than those to the NH2-terminal side (26). The peptide sequence containing Ser363 in the COOH terminus of Cx45.6 is: E[358]EEVS[363]DEVE[367], which satisfies both criteria as potential consensus sequences for CKII. In addition, this peptide sequence has a Glu residue at the −3 position NH2-terminal to the target Ser363 and satisfies the CKI consensus sequence requirement for an acidic residue, three residues to the amino-terminal side of the target Ser/Thr (26). Although CKI and CKII have distinctive substrate preferences, several proteins are reported to be phosphorylated by both CKII and CKI in vitro at the same site (38). To examine whether Ser363 in Cx45.6 can be phosphorylated by CKI or/and CKII, we performed in vitro phosphorylation by CKI/CKII with the peptide RR[358]EEVS[363]DEVE[367]. Phosphorylation consensus peptides for CKI (RR[KDLHDDEEDEAMSITA]) and CKII (RR[REEETTEE]), as well as the peptide containing PKα sequence (CG[TPYE]ALPEIIR), were used as controls. The phosphorylation level of each peptide phosphorylated by CKI/CKII was evaluated compared with the non-peptide control. Table I shows that the peptide (Cx45.6, amino acids, 358–367) was significantly more phosphorylated by CKII compared with the non-peptide control. GST was not phosphorylated by CKII, although autophosphorylation of CKII was observed (Fig. 2A, lane 5). Both the CKII subunits migrating mainly at 28 and 35 kDa (Fig. 2A, lane 4) showed that a single spot from endogenous Cx45.6 migrated to the same position as that of GST-Cx45.6F (Fig. 2A, lane 6), GST-Cx45.6F was highly phosphorylated by CKII and migrated mainly at 28 and 35 kDa (Fig. 2A, lane 4), GST-Cx45.6F (lane 6), or dH2O (lane 5) were reacted with CKII in the presence of [γ-32P]ATP and the products were separated on 12% SDS-PAGE. The lenses dissected from day 11 chick embryos in culture medium were labeled overnight with [32P]orthophosphate, lysed, immunoprecipitated with anti-Cx45.6 antibody and digested with TPCK-trypsin. The tryptic peptides from phosphorylated Cx45.6, GST-Cx45.6F, or mixture of both were applied to two-dimensional TLC at pH 1.9.

In Vivo and in Vitro Phosphorylation Profile Comparison Suggested the Direct Phosphorylation of Cx45.6 by CKII—To verify that full-length Cx45.6 in its native state can be phosphorylated by CKII, lens membranes were used as a substrate for in vitro phosphorylation reaction with CKII, followed by immunoprecipitation with affinity purified anti-Cx45.6 antibody. The resulting immunoprecipitates were separated on SDS-PAGE and detected by autoradiography as shown in Fig. 2A. CKII catalyzed the phosphorylation of Cx45.6 in lens membranes as shown by the dominant phosphorylated band migrating around 56 kDa (Fig. 2A, lane 1, solid arrowhead). No phosphorylated bands were detected in the immunoprecipitates from the kinase reaction with CKII plus [γ-32P]ATP (Fig. 2A, lane 2) without lens membrane or lens membrane plus [γ-32P]ATP without CKII (Fig. 2A, lane 3). These results showed that Cx45.6 in chick lens membrane could be phosphorylated in vitro by CKII.

To further confirm the phosphorylation of Cx45.6 at its COOH terminus, a fusion protein containing GST linked with the entire COOH-terminal tail of Cx45.6 (GST-Cx45.6F) was used as a substrate for the in vitro kinase reaction with CKII. GST-Cx45.6F was highly phosphorylated by CKII and migrated to the position around 60 kDa (Fig. 2A, lane 4, solid arrowhead), which was different from the autophosphorylated CKII subunits migrating mainly at 28 and 35 kDa (Fig. 2A, lane 5). GST was not phosphorylated by CKII, although autophosphorylation of CKII was observed (Fig. 2A, lane 6).

To determine whether Cx45.6 is phosphorylated by CKII in vivo on the same sites as the in vitro phosphorylation, two-dimensional tryptic phosphopeptide mapping was performed. Endogenous full-length phosphorylated Cx45.6 was revealed from [32P]-labeled lens organ cultures by immunoprecipitation with anti-Cx45.6 antibody (Fig. 2A, lane 7). Both the in vitro phosphorylated Cx45.6 and the in vitro phosphorylated fusion proteins were resolved by SDS-PAGE (Fig. 2A, lanes 4 and 7, respectively) and the appropriate bands were excised, digested with trypsin, and analyzed using two-dimensional TLC. Comparisons of two-dimensional TLC phosphopeptide profiles showed that a single spot from endogenous Cx45.6 migrated to the same position as that of GST-Cx45.6F (Fig. 2B, left and

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**TABLE I**

Comparison of synthetic peptide phosphorylation by CKI and CKII

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kinase</th>
<th>-Fold of increased radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx45.6 peptide (RRREEEVS[358]DEVE)</td>
<td>CKI</td>
<td>4.1 ± 1.5</td>
</tr>
<tr>
<td>CKI-specific peptide (RRKDLHDDEEDEAMSITA)</td>
<td>CKI</td>
<td>501.6 ± 139</td>
</tr>
<tr>
<td>CKII-specific peptide (RRREETTEE)</td>
<td>CKI</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>Cx45.6 peptide (RRREEEVS[363]DEVE)</td>
<td>CKII</td>
<td>106.1 ± 5.9</td>
</tr>
<tr>
<td>CKII-specific peptide (RRREETTEE)</td>
<td>CKII</td>
<td>228.1 ± 7.9</td>
</tr>
<tr>
<td>PKα peptide (CG[TPYE]ALPEIIR)</td>
<td>CKII</td>
<td>0.08 ± 0.1</td>
</tr>
<tr>
<td>Cx45.6 peptide (RRREEEVS[363]DEVE) + heparin</td>
<td>CKII</td>
<td>1.9 ± 0.06</td>
</tr>
</tbody>
</table>
TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fold of increased activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKII peptide (RRREEETEEE)</td>
<td>32.8 ± 0.98</td>
</tr>
<tr>
<td>PKCa peptide (CGTPEYLAPEIRR)</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>CKII peptide (RRREEETEEE) without lysate</td>
<td>0.45 ± 0.005</td>
</tr>
</tbody>
</table>

Presence of CKII Activities in Chick Lens Lysate—We examined the presence of CKII activity in the lens lysate because CKII in lens has never been demonstrated. Embryonic day 17 lens lysate was isolated and the CKII activity was assayed by its ability to phosphorylate a specific CKII substrate consensus peptide (RRREEETEEE). Table II shows that under the specific reaction conditions, the phosphorylation of CKII-specific substrate peptide by lens lysate was more than 30-fold greater compared with lens lysate alone, lens lysate plus PKCa peptide (CGTPEYLAPEIRR), or peptide alone without lysate. The phosphorylation reaction by lens lysate was inhibited by a CKII-specific inhibitor, heparin (Fig. 3). The degree of the inhibition was associated with the increased concentration of the applied heparin. The apparent 50% inhibition was estimated to occur at 2 μg/ml, which is comparable to the previously reported value (39) under similar conditions. These results are indicative of the CKII specific activity in the lens lysate.

Phosphorylation at Ser363 by CKII-Facilitated Cx45.6 Degradation—In order to analyze the function of Ser363 phosphorylation of Cx45.6, overexpression of exogenous wild type and mutant (Ser363 → Ala) Cx45.6 in lens fiber-like cells is required. Currently, no cell lines for lens fiber cells are established. The traditional transfection approaches for the expression of the exogenous proteins in the primary cultures are known to be difficult and inefficient. We have developed a retroviral approach that can efficiently express lens connexins both in vivo and in the primary cultures (40).

Retroviral constructs were prepared containing wild type and mutant (Ser363 → Ala) Cx45.6. COOH termini of both constructs were tagged with FLAG sequence to distinguish them from the endogenous connexins. The high titer retroviruses containing Cx45.6 or Cx45.6 (Ser363 → Ala) were infected into chick lens primary cell cultures. After a week, when the cells differentiated and formed the lens fiber-like lentoid structures (11), the expressions of exogenous wild type (Fig. 4A, lanes 2 and 4) and mutant Cx45.6 (Fig. 4A, lanes 1 and 3) were demonstrated by Western blots with anti-Cx45.6 (Fig. 4A, lanes 1 and 2) and anti-FLAG antibodies (Fig. 4A, lanes 3 and 4). The upper most bands detected by the Cx45.6 antibody (Fig. 4A, lanes 1 and 2) were exogenously expressed Cx45.6 and its mutant. The specific expressions of exogenous Cx45.6 constructs were further examined by indirect immunofluorescence studies (Fig. 4B). The immunofluorescence results showed that the Cx45.6 and its mutant were localized in the lentoid structures detected by either anti-Cx45.6 or the FLAG antibody. The results confirmed that each RCAS(A) that contained correct connexin inserts with COOH terminus tagged FLAG sequence could be expressed in the fiber-like lentoid cells of the lens primary cultures.

Pulse-chase experiments were performed to study the kinetics and the turnover of the wild type and mutant Cx45.6. Cells were labeled with 35S methionine for 3 h followed by 0, 6-, 12-h chase periods in non-radioactive complete culture medium. The cell lysates were immunoprecipitated with affinity purified anti-Cx45.6 antibody (Fig. 5). After 3 h labeling, Cx45.6 was detected as a triplet in which the top two bands were less distinctive due to the width of the signals (Fig. 5, lanes 1 and 4). The fastest migrating band of Cx45.6 was shown previously to be the most phosphorylated form (13, 17). With a longer chase time of 6 and 12 h (Fig. 5, lanes 2, 3, 5, and 6), wild type Cx45.6 appeared to diminish much faster compared with mutant Cx45.6. Analysis of the protein band intensity gave an estimate of the half-life of 3 h, one-half hour longer than that of the wild type Cx45.6. After 12 h, the intensity of wild type Cx45.6 was only one-fourth of the mutant Cx45.6. The results showed that the mutation of Ser363 → Ala stabilized Cx45.6, indicating that the phosphorylation at Ser363 of Cx45.6 is likely to participate in the degradation process of Cx45.6.
Connexin Phosphorylation by Casein Kinase II

FIG. 5. Analysis of the half-life of wild type Cx45.6 and mutant Cx45.6 (Ser363 → Ala) expressed in lens cell primary cultures by retroviral infection. The cells were labeled with [35S]methionine for 3 h and chased for 0 h (lanes 1 and 4), 6 h (lanes 2 and 5), or 12 h (lanes 3 and 6). The cultures were lysed, immunoprecipitated by affinity purified anti-Cx45.6 antibody, separated by SDS-PAGE, and detected by autoradiography.

**DISCUSSION**

In this report, we have shown that the lens fiber Cx45.6 is phosphorylated in vivo on Ser363 at the COOH terminus and that this phosphorylation is likely to be mediated by CKII. The involvement of CKII can be summarized by the following experimental evidence. 1) A synthetic peptide corresponding to residues 356–367 of Cx45.6 was in vitro phosphorylated by CKII, and not by CKI. 2) Cx45.6 isolated from embryonic lens membranes and fusion protein containing COOH terminus of CKII, and not by CKI. 2) Cx45.6 isolated from embryonic lens experimentally. 1) A synthetic peptide corresponding to nonspecific site, we attributed these two additional phosphopeptides to Since there is no additional CKII phosphorylation consensus sequences. We thank D. Adan-Rice for technical assistance and members of J. X. Jiang’s laboratory for critical reading of the manuscript.

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163–175
43. Deleted in proof
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