Connexin (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 Ser\textsuperscript{363} site in the carboxyl (COOH) terminus of Cx45.6 was phosphorylated and that this site is within casein kinase (CK) II consensus sequence, although showing some similarity to CKI sequence. The peptide containing Ser\textsuperscript{363} 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 (Ser\textsuperscript{363} → 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 Ser\textsuperscript{363} 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). PKC\textsuperscript{I} 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 in vitro and in vivo analyses confirmed that CX45.6 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, NY) and Tyson Hatchery (Gonzales, TX). CKII, heparin, and CKI consensus peptide (RRKKHIDDEEDEAMSA) were obtained from Sigma. CKI was purchased from Promega (Madison, WI). The peptide derived from CX45.6 (Ser\(^{358}\)RRREEEVYS\(^{363}\)DEVE\(^{367}\)), and CKII consensus peptide (RRREEEETEEE) 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 Galveston. PKC-peptide, (CGTPEYLAPEIRR) was kindly provided by Dr. F. Liu at the University of Texas Health Science Center at Galveston. 

**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 (12). Briefly, lenses loaded with \(^{35}S\)-labeled samples were processed for fluorography by FLAG antisera were from Eastman Kodak (Rochester, NY). For gels loaded with \(^{32}P\)-labeled samples, intensifying screens were used. The substrate peptides in CKII reaction included RRREEEETEEE (CKII, consensus peptide), or CGTPEYLAPEIRR (PKC\(\alpha\) peptide). CKII buffer contained 10 mM MgCl\(_2\), 50 mM MOPS (pH 7.0), 150 mM NaCl, and 2.5 \(\mu\)Ci of \[^{32}P\]ATP. The substrate peptides in CKII reaction included a peptide of CX45.6 amino acids 358–367, RRKDHLDEEDEAMSITA (CKI, consensus peptide) or CKII consensus peptide. After incubation at 37 °C for 30 min, the reaction was terminated by 10% trichloroacetic acid (w/v). The sample was kept on ice for 10 min prior to centrifugation at 13,000 \(\times\) g for 5 min. Fifteen microliter supernatant was spotted on a 2.5-cm Whatman P81 phosphocellulose paper and then washed extensively with 0.75% phosphoric acid (w/v) 5 min each three times and washed with acetone once. When dry, the paper was transferred to a scintillation vial to measure radioactivity using a Packard counter (Meriden, CT).

**Mapping and Sequencing of the Cx45.6 Phosphorylation Sites by Nanoelectrospray Mass Spectrometry—**SDS-PAGE purified CX45.6 bands were excised and digested in situ overnight (for protocol, see Ref. 29) in Eppendorf tubes at 37 °C with modified trypsin (Promega, Madison, WI) at an approximate ratio of 10:1 (protein:enzyme). A fraction of each digest was purified over Poros R2 50 (Perseptive Biosystems, Framingham, MA) microcolumns and eluted into nanospray needles (Protana, Denmark) with 5% formic acid and 50% acetonitrile (for details, see Ref. 29). Nanoelectrospray mass spectra were acquired on a Finnigan LCQ ion trap mass spectrometer (San Jose, CA) equipped with an ESI ion source. The standard spray needle assembly was replaced with a nanospray source of in-house design (30). Solutions were sprayed with a needle potential of about +0.8 kV. Tandem MS spectra were acquired with relative CID of between 20 and 35%.

**In Vitro 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. 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 in vitro and in vivo analyses confirmed that CX45.6 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.

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**Materials**—Fertilized chicken eggs were obtained from SPAFAS (Ronkonkoma, NY) and Tyson Hatchery (Gonzales, TX). CKII, heparin, and CKI consensus peptide (RRKKHIDDEEDEAMSA) were obtained from Sigma. CKI was purchased from Promega (Madison, WI). The peptide derived from CX45.6 (Ser\(^{358}\)RRREEEVYS\(^{363}\)DEVE\(^{367}\)), and CKII consensus peptide (RRREEEETEEE) 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 Galveston. PKC-peptide, (CGTPEYLAPEIRR) was kindly provided by Dr. F. Liu at the University of Texas Health Science Center at Galveston. 

**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 (12). Briefly, lenses loaded with \(^{35}S\)-labeled samples were processed for fluorography by incubation in 1% salsicylic acid for 30 min prior to drying. The protein bands were quantified by Image-pro Plus Program (Media Cybernetic, Rochester, NY). For gels loaded with \(^{32}P\)-labeled samples, intensifying screens were used. Western blots of proteins were performed by probing with either anti-FLAG or affinity-purified anti-CX45.6 antibody. Primary antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibody.

**Preparation of the Phosphorylated CX45.6 for the Phosphorylation Site Mapping**—Approximately 550 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 modified procedures as described previously (12). Briefly, lenses were lysed and centrifuged at 100,000 \(\times\) g for 30 min at 4 °C. The pellets were resuspended in 20 mM prechilled NaOH to eliminate periplasmic proteins and centrifuged at 100,000 \(\times\) g for 30 min at 4 °C. The supernatant was then centrifuged again at 100,000 \(\times\) g for 30 min at 4 °C. The resulting supernatants were further fractionated into 10% SDS-PAGE. The labeled bands were identified by autoradiography and excised from dried gels. The gel slices were washed with 3× volumes of 50% ethanol, 100 mM NaCl, 2.5 mM 8-mercaptoethanol, 50 mM glycine, and 0.1% SDS for 15 min each. The gel slices were then dried completely in speed vacuum and rehydrated into 200 μL of TPK-treated trypsin (10 mg/ml) in 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 dH\(_2\)O. The final tryptic digested products were resuspended in electrophoresis buffer and separated on two-
Connexin Phosphorylation by Casein Kinase II

dimensional TLC using the procedure described by Boyle et al. (34). The first dimension on TLC was separated by electrophoresis at a buffer pH of 1.9 and the second dimension by chromatography in phosphochromatography buffer.

Analysis of the CKII Activity in Lens Lysate—The CKII activity assay was initiated by the addition of embryonic day 16 lens lysate containing 5–10 μg of proteins to CKII phosphorylation buffer (20 μCi of \( \gamma^{32}\text{P} \text{ATP}, 10 \text{mm MglCl}, 50 \text{mm MOPS}, \text{pH} 7.0, 150 \text{mm NaCl}, 2 \text{mm Na}_{2} \text{VO}_4, 2 \text{mm EDTA}, \text{and} 25 \text{mm} \beta\text{-glycerolphosphate} \)) plus or minus 2 mM peptides. The peptides used for the assay included the above described CKII consensus peptide and PKCζ peptide. Another control experiment included CKII phosphorylation buffer and CKII consensus peptide without lens lysate. The specific inhibition of CKII activity in chick lenses by heparin was done by adding various concentrations of heparin to lens lysate dissolved in CKII phosphorylation buffer. The remaining procedures for CKII activity assay were exactly the same as described above for in vitro phosphorylation of synthetic peptides.

Preparation of the Retroviral Constructs and High-titer Retroviruses—Preparation of retroviral constructs and high-titer viruses was based on the published procedure with modifications (35). Briefly, DNA fragments containing wild type and mutant Cx45.6 (Ser363 → Ala) were made by polymerase chain reaction. The codon (AGC) encoding Ser363 was changed to (GCC) encoding Ala in one of the two oligonucleotide primer sequences required for mutant Cx45.6 (Ser → Ala) synthesis. COOH termini of both wild type and mutant Cx45.6 were epitope-tagged with FLAG sequences to distinguish the exogenous from endogenous connexins. Nohl/EcoRI restriction sites were created at NH2- and COOH-terminal ends of the DNA fragments, respectively. The polymerase chain reaction products were subcloned into an adapter vector, ClaI2NCO, which shared the same Clal restriction site as retroviral vector, RCAS(A) (36). To ensure the correct sequence of the constructs, the DNA fragments were sequenced at the Institutional DNA Sequencing Facility. Connexin fragments were isolated following digestion with ClaI and subcloned into Clal-linearized RCAS(A). The constructs with the correct inserts were selected and transfected into retrovirus packaging cells, chick embryonic fibroblast and the expression of Cx45.6 was detected by Western blot with Cx45.6-FLAG antibody (Eastman Kodak) and affinity purified Cx45.6 antibody. When the transfected chick embryonic fibroblast cells became confluent, they were split in the following order: 60-mm plate → 100-mm plate → 150-mm plate → five 150-mm plates. The culture media were collected consecutively for 3 days from five 150-mm dishes when the cells became confluent. The retroviruses in the culture medium were concentrated by centrifugation at 72,000 g. The supernatants were used to infect the cells at a multiplicity of infection of 100–300. The method described previously (19) was followed when the cells became confluent. The retroviruses in the culture medium were concentrated by centrifugation at 72,000 g. The supernatants were used to infect the cells at a multiplicity of infection of 100–300.

Preparation of Embryonic Lens Primary Cultures, Retroviral Infection, and Pulse-Clase Analysis—The lens primary cultures were prepared following the procedure described previously (19). Briefly, chick lenses dissected from day 11 embryos were washed in buffer containing 140 mM NaCl, 5 mM KCl, 0.7 mM NaHPO4, 5 mM glucose, 25 mM Tris-Cl (pH 7.4). The intact lenses freed of digested extracapsular tissues were rinsed in culture medium (Dulbecco’s modified Eagle’s medium supplemented with 0.5 mM methionine and 10% fetal calf serum). 1×106 cells were plated in 35-mm tissue culture plates. High-titer retroviruses (5 μl/dish) carrying either wild type Cx45.6 or mutant Cx45.6 (Ser363 → Ala) DNA were used to infect monolayer primary lens epithelial cultures the next day. The cultures were fed daily and used for labeling after a week when cells became 80–90% confluent and formed lens fiber-like lenticoid structures (11). The cultures were rinsed three times with the labeling medium (methionine-free Dulbecco’s modified Eagle’s medium with 5% dialyzed fetal calf serum, 20 μM methionine) and incubated in 1 ml above labeling medium plus 0.5 μCi of [35S]methionine for 3 h. Then the cultures were rinsed with complete medium (Dulbecco’s modified Eagle’s medium supplemented with 0.5 μM methionine and 10% fetal calf serum) and incubated in the non-radioactive medium for various chase periods.

RESULTS

Identification of in Vivo Cx45.6 Phosphorylation Site at Ser363—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)) 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, 1305.4 corresponding to doubly and singly charged phosphopeptides, 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 (m/z 1354.5, K356AEEEVVSpDEVEGPSAPAELATDVR). Sufficient number of complementary B ions (NH2 terminus derived fragment ions) and Y ions (COOH terminus derived fragment ions) were observed to assign the site of phosphorylation to Ser363. 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 NH2 terminus while the superscripts indicate the charge state of each peptide. Tryptin autolysis peptides are indicated by an asterisk. Mass spectrum (B) of T37–38(Sp) phosphopeptide (m/z 1354.5, K356AEEEVVSpDEVEGPSAPAELATDVR). Sufficient number of complementary B ions (NH2 terminus derived fragment ions) and Y ions (COOH terminus derived fragment ions) were observed to assign the site of phosphorylation to Ser363. 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 Peptide Corresponding 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: E358EEVVS363DEVE367, 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 RRREEEVVS363DEVE from the chick lens membrane by CKII and comparison of tryptic phosphopeptide mapping of Cx45.6 phosphorylation in vitro and in vivo. A, embryonic day 11 chick lens membranes were reacted with CKII and [γ32P]ATP (lane 1) or [γ32P]ATP alone (lane 3). CKII was also incubated with [γ32P]ATP in the absence of lens membranes (lane 2). All reaction products were immunoprecipitated with anti-Cx45.6 antibody and separated on 10% SDS-PAGE. GST-Cx45.6F (lane 4), GST (lane 6), or dH2O (lane 5) were reacted with CKII in the presence of [γ32P]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 detected on 10% SDS-PAGE (lane 7). B, [32P]-labeled phosphorylated Cx45.6 and GST-Cx45.6F were separated by 10% SDS-PAGE, excised, and digested with TPKC-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.

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>
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<tbody>
<tr>
<td>Cx45.6 peptide (RRREEEVVS363DEVE)</td>
<td>CKI</td>
<td>4.1 ± 1.5</td>
</tr>
<tr>
<td>CKI-specific peptide (RRKLDHDEEAMGISA)</td>
<td>CKI</td>
<td>501.6 ± 139</td>
</tr>
<tr>
<td>CKII-specific peptide (RRREEETEEE)</td>
<td>CKII</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>Cx45.6 peptide (RRREEEVVS363DEVE)</td>
<td>CKII</td>
<td>106.1 ± 5.9</td>
</tr>
<tr>
<td>CKII-specific peptide (RRREEETEEE)</td>
<td>CKII</td>
<td>228.1 ± 7.9</td>
</tr>
<tr>
<td>PKCa peptide (CGTPEYLAPRVE)</td>
<td>CKII</td>
<td>0.08 ± 0.1</td>
</tr>
<tr>
<td>Cx45.6 peptide (RRREEEVVS363DEVE) + heparin</td>
<td>CKII</td>
<td>1.9 ± 0.06</td>
</tr>
</tbody>
</table>

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 [γ32P]ATP (Fig. 2A, lane 2) without lens membrane or lens membrane plus [γ32P]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 Cx45.6 antibody (Fig. 2A, lane 7). Both the in vivo 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
center panels). Mixing both samples showed co-migration of the two peptides (Fig. 2B, right panel). The results suggest that CKI is likely to phosphorylate Cx45.6 in vivo.

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 PKCα 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 expression 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 antibody. The expression of both constructs in lens primary cultures were examined by indirect immunofluorescence (Fig. 5). The fixed primary culture cells were labeled with Cx45.6 or FLAG antibody and detected by rhodamine-conjugated corresponding secondary antibodies.

TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fold of increased activity</th>
</tr>
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<tbody>
<tr>
<td>CKII peptide (RRREEETEEE)</td>
<td>32.8 ± 0.98</td>
</tr>
<tr>
<td>PKCα 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>

Fig. 3. Heparin inhibited phosphorylation of CKII-specific peptide catalyzed by embryonic lens lysate. Embryonic day 16 chick lens lysate containing equal amounts proteins were utilized to phosphorylate CKII substrate peptide (RRREEETEEE) in the presence of various concentrations of heparin. Control experiments were performed by incubating [γ-32P]ATP with lens lysate in the absence of CKII peptide. The phosphorylation activities were presented by dividing the data of peptide reaction from that of non-peptide control.

Fig. 4. Expression of exogenous Cx45.6 constructs in primary lens cultures by retroviral infection. The primary cultures of embryonic lenses were infected by retroviruses containing RCAS(A)-Cx45.6 and RCAS(A)-Cx45.6 (Ser363 → Ala) at the second day after plating. After 1 week of culture, when lentoids were formed, Western blots were performed (A) with cell lysate of lens primary cultures infected with retrovirus containing RCAS-Cx45.6 (Ser363 → Ala) (lanes 1 and 3) or RCAS-Cx45.6 (lanes 2 and 4) and blotted by anti-Cx45.6 (lanes 1 and 2) or anti-FLAG (lanes 3 and 4) antibody. The expression of both constructs in lens primary cultures were examined by indirect immunofluorescence (B). The fixed primary culture cells were labeled with Cx45.6 or FLAG antibody and detected by rhodamine-conjugated corresponding secondary antibodies.

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 mutant Cx45.6 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.
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 355 ~ 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 Cx45.6 could be in vitro phosphorylated by CKII. 3) Two-dimensional phosphopeptide mapping showed an in vitro phosphorylated peptide sharing the same migration spot as an in vivo phosphopeptide as its endogenous counterpart (40). In addition, exogenous lens connexins were introduced by retroviral infection and were expressed at the same regions as their endogenous counterparts (40). In addition, exogenous Cx45.6 is phosphorylated similarly as the endogenous form, although the unphosphorylated upper forms of exogenous Cx45.6 are the predominant forms.

Using the PEST search utility program, which identifies sequence domains which are rich in proline, glutamic acid, serine, and threonine residues (PEST) (36), the sequence of amino acid residues from 356 to 380 of Cx45.6 containing Ser363 was identified as a potential PEST domain. It has been reported that the CKII phosphorylation sites of several proteins were localized within a PEST domain (35, 41). The PEST domain has been found to be associated with a rapid protein turnover (39), also CKII phosphorylation has been reported in a number of cases to regulate protein degradation (35, 41) such as IκBα. The degradation of connexins seems to employ either lysosome or proteasome pathway (43, 44). Phosphorylation of the Ser363 of Cx45.6 is localized within the PEST domain. Since most proteins containing PEST domain undergo proteasome degradation pathway, Cx45.6 degradation induced by Ser363 phosphorylation is likely to undergo this pathway.

Phosphorylation induced connexin degradation has significant functional implications in the regulation of gap junctions in vivo. MP70, ovine ortholog of Cx45.6, is cleaved in the mature fibers located close to the center of the lens (45, 46). The gating of gap junctions formed by the truncated MP70 displays a significant reduction in pH sensitivity, which reveals how the fiber cells in the central lens regions remain functional despite the acidic environment caused by elevated lactate levels (47).

In addition, the phosphorylation of the lens fiber connexins increases in the direct correlation to the development of the lens and the accumulation of the more mature fibers (24). Therefore, our study in conjunction with previous evidence suggests a novel regulatory mechanism of the gap junctions in vivo: the phosphorylation of the connexin by CKII in the mature lens fibers leads to its degradation by proteases, and the gap junctions formed by truncated connexin possess a unique channel physiology which accommodates the metabolic needs of cells and tissues.

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