Calmodulin (CaM) mediates intracellular Ca\(^{2+}\) by regulating cellular enzymes in a Ca\(^{2+}\)-dependent manner. A variety of in vitro techniques in many biological systems have been used to implicate CaM in nuclear activities. The nucleus contains several CaM-binding proteins (1) including protein kinases and phosphatases (2, 3). Ca\(^{2+}\)-CaM is capable of directly binding translocation factors containing basic helix-loop-helix domains and can differentially inhibit in vitro transcription (4). In vitro, CaM kinase II can phosphorylate the CAM response element-binding protein (5, 6). Attenuation of interleukin-2 transcription occurs in T-cells expressing a constitutively active CaM kinase II (7). Inhibitors of calcineurin, a Ca\(^{2+}\)-CaM-dependent phosphatase, alter the in vitro transcription of many early response genes (8, 9).

Proteolytic mapping of a major CaM target enzyme, skeletal muscle myosin light chain kinase (MLCK), was used to identify a peptide that binds Ca\(^{2+}\)-CaM with an affinity (10\(^{-9}\) M) similar to that of the native competitive inhibitor of CaM-dependent enzymes. In the present study, the MLCK CaM binding domain was used to design a synthetic concanavalin gene that would target and functionally neutralize CaM in the nuclei of cultured cells and in lung epithelium of transgenic mice.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression of MLCK Peptide Gene in E. coli—A synthetic gene (72 bp) containing a ATG initiation codon and Xmal restriction enzyme sites at each end was designed to encode the rabbit skeletal muscle MLCK CaM-binding sequence, KRRWKNFIA- VSAANRRFKK. The respective coding and complimentary oligonucleotides were annealed and cloned into the Xmal site of pBluescript SK (Stratagene) using T4 DNA ligase. Proper orientation of the MLCK peptide gene was confirmed by dyeoxy DNA sequencing (United States Biochemical Corp.). A BamHI-EcoRI fragment containing the coding sequence was released from pBSK and fused to the 3' end of the glutathione S-transferase (GST) gene using the BamHI and EcoRI sites of the bacterial expression vector pGEX-2T (Pharmacia Biotech Inc.).

The GST-MLCK peptide fusion protein (30.6 kDa) was induced in E. coli (SURE cells, strain 835) containing the pBluescript SK plasmid with 100 \mu g of a mixture containing equal amounts of 4-bipyrrolidonyl-\beta-N-succinimidyl at 37°C in 50 mM HEPES (pH 7.5) containing 1 mM magnesium acetate, 10 mg/ml bovine serum albumin, 0.1 mM [\(\gamma\)-\(^{32}\)P]ATP (DuPont NEN), 0.1 \mu g of CaM kinase II, and 0.5 \mu g of myelin basic protein, with or without 0.5 \mu g of calmodulin as indicated. Synthetic MLCK peptide or purified GST-MLCK peptide fusion protein at the indicated concentrations were added. The reaction was terminated after 30 s with 10% trichloroacetic acid. The mixture was centrifuged at 12,000 rpm for 15 min and the resulting pellets dissolved in 150 \mu l of SDS sample buffer and separated by 12% SDS-PAGE. The gel was dried and autoradiographed.

Production and Affinity Purification of Anti-MLCK Peptide Antibodies—Initial injections into four individual rabbits were performed using KRRWKNFIAVSAANRRFKK coupled to keyhole limpet hemocyanin (KLH, Sigma). The quality of the peptide was confirmed by fast atom mass spectroscopy. Six milligrams of the peptide were mixed with an equal weight of KLH and suspended in distilled water (pH 5.0); 5 mg of carbodiimide (Pierce) was dissolved in the mixture. A second 6 mg mixture of the peptide and KLH was suspended in 125 mg borate (pH 8.4) containing 75 mg NaCl and 5 mg of dimethylsulfoxide. Both mixtures were allowed to react overnight in the dark at room temperature. The reactions were terminated by the addition of 100 \mu l of 0.1 M ethanamine. For the first injection, each rabbit received a 2-mg peptide equivalent of the mixture emulsified in complete Freund’s adjuvant. The third and fourth injections were on days 45 and 60, respectively. Each boost contained 1 mg of free peptide in incomplete Freund’s adjuvant. The animals were bled on day 75 and every 2 weeks thereafter.

Affinity columns were constructed using 5 mg of the free peptide dissolved in 125 mg borate (pH 8.4) containing 75 mg NaCl and 5 ml of E. coli (SURE) cell extract (Stratagene) with 100 \mu g of a mixture containing equal amounts of 4-bipyrrolidonyl-\beta-N-succinimidyl at 37°C in 50 mM HEPES (pH 7.5) containing 10 mM magnesium acetate, 10 mg/ml bovine serum albumin, 0.1 mM [\(\gamma\)-\(^{32}\)P]ATP (Dupont NEN), 0.1 \mu g of CaM kinase II, and 0.5 \mu g of myelin basic protein, with or without 0.5 \mu g of calmodulin as indicated. Synthetic MLCK peptide or purified GST-MLCK peptide fusion protein at the indicated concentrations were added. The reaction was terminated after 30 s with 10% trichloroacetic acid. The mixture was centrifuged at 12,000 rpm for 15 min and the resulting pellets dissolved in 150 \mu l of SDS sample buffer and separated by 12% SDS-PAGE. The gel was dried and autoradiographed.

Expression and Localization of the MLCK Peptide Gene in Mammalian COS-7 Cells—5'-CGGATTCTTAGAAGAGAAGATGG-3' and 5'-ACTACCGAGCTTCGAGAATTCGTT-3' were designed to incorporate an AscI restriction enzyme site (CTCGGG) at each end of the monomer gene in pBluescript SK using the polymerase chain reaction (PCR). Following Ampligol digestion, the amplified monomer genes (66 bp) were self-ligated using T4 DNA ligase in a head-to-tail manner. The ligation mixture was blunted-ended using Klenow and cloned into the Smal site of pBluescript STR (Stratagene). Clones that contained differ-
pressed, would eliminate the cellular function of Ca2+-binding sequence to design a gene, which, when ex-

pressed in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum at 37°C in a 5% CO2 humidified incubator. Recombinant DNA (20 ng) was purified using a Qiagen Maxi Kit and was transiently transfected into COS-7 cells by the calcium-phosphate precipitation method (31). Transfected cells were grown on 22 mm coverslips. At 24, 48, or 72 h after transfection, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. The fixed cells were incubated for 1 h with an affinity-purified rabbit anti-MLCK peptide antibody, washed with phosphate-buffered saline, and incubated with fluorescein-conjugated goat anti-rabbit IgG. Double-stained cells were also incubated with 1 h with 10% normal rabbit serum, followed by affinity-purified sheep anti-calmodulin antibody and CY3-conjugated sheep anti-rabbit immunoglobulin. The coverslips were then mounted and photographed using a Zeiss Axiovert 200 Ektachrome film with a Nikon Microflex UFX exposure attachment mounted on a Nikon Optiphot epifluorescence microscope and the appropriate Nikon fluorescence filter cube.

Targeted Expression of MLCK Peptide Gene to the Lung Epithelium of Transgenic Mice—The transgene is composed of the 3.7-kb HindII-HindIII fragment of human SP-C promoter, 318-bp Nhel-XhoI fragment of a four-repeat MLCK peptide gene, and 850-bp XhoI-BamHII fragment of SV40 intervening sequence and polyadenylation signal. The 4.8-kb transgene was excised from pBluescript KS by SalI and NotI digestion, separated on 1.0% agarose gel, and purified by GeneClean (Bio101 Inc.). This DNA was dissolved in 0.1 M Tris (pH 8.3) and 0.1 mM EDTA and microinjected into donor eggs of mouse strain FVB/N by the University of Cincinnati Transgenic Animal Core Facility. Founder mice were identified by PCR amplification of DNA isolated from tail-clippings using primer 5'-AGCTGGATATCGAATTCCTG-3' (specific for human SP-C) and the 3' primer, 5'-GCAAGCAAGATCCTTCACTG-3' (specific for the 3' untranslated region of SV40). The PCR product was a 703-bp fragment of the MLCK peptide transgene. Four surviving founder mice were identified from 83 pups delivered by surrogate mothers. Embryos obtained from the X-linked transgenic females were harvested and fixed in 4% paraformaldehyde for 48 h at 4°C. The fixed tissues were then embedded in paraffin, and 4-μm cross-sections were cut. Deparaffinized sections were incubated with 2 μg of the respective affinity-purified rabbit anti-MLCK peptide antibody for 2 h, washed three times in wash buffer and blocked in phosphate-buffered saline, incubated with fluorescein-conjugated goat anti-rabbit IgG for 1 h, and washed again. Sections mounted in glycerol/phosphate-buffered saline (9:1) containing 0.1% p-phenylenediamine were viewed on a Nikon epifluorescence microscope.

RESULTS AND DISCUSSION

We used the rabbit skeletal muscle MLCK 19-amino acid CaM-binding sequence to design a gene, which, when expressed, would eliminate the cellular function of Ca2⁺/CaM by competing with endogenous target proteins. In order to test whether the synthetic gene product would inhibit CaM, the MLCK peptide gene was expressed in bacteria as a fusion protein. The fusion protein, like the chemically synthesized peptide, inhibits Ca2⁺/CaM-dependent protein kinase II activity in a concentration-dependent manner (Fig. 1). The sequence of the first two repeats of the concanamer is: MKRRWKKNFIAVAANRFKFKLG. This sequence contains several basic amino acid clusters and the bipartite lysine-lysine motif, KK . . . . . . KK (underlined). These are potential nuclear localization signals common in many karyophilic proteins such as the SV40 T-antigen and transcription factors (16). In order to evaluate the cellular targeting of the CaM inhibitor gene and its effects in intact cells, COS-7 cells were transiently transfected with the concanamer gene. The cells were examined by indirect immunofluorescence using affinity purified antibody produced against the inhibitor peptide. As shown in Fig. 2a, approximately 10% of the cells expressed the MLCK peptide concanamer, which was concentrated in the nuclei of transfected cells (Fig. 2a, c and d). During early stages of expression, the peptide staining pattern was “donut-like” (Fig. 2e). By 72 h, the nuclei became extremely large and multi-lobed, and contained condensed chromatin indicating disruption of nuclear function (Fig. 2f). It did not appear that the MLCK peptide-transfected cells entered mitosis. A splicing isoform of CaM kinase II contains the nuclear targeting sequence, KKRK, and is localized in the nucleus (3). Microinjection of a CaM kinase II inhibitory peptide into sea urchin eggs causes a delay in nuclear envelope breakdown (17). Targeted overexpression of the MLCK peptide in the nucleus may inhibit CaM kinase II activity preventing cells from entering mitosis. Double immunostaining of transfected cells demonstrates coincident localization of CaM and the MLCK peptide in the nucleus. CaM is also present throughout the cytoplasm, indicating that only nuclear functions of CaM were being neutralized (Fig. 2, g and h).

In order to evaluate the consequences of expression of the MLCK peptide during cell growth and differentiation, the CaM inhibitor gene was targeted to a specific cell type in transgenic mice. Due to the potent inhibitory effects of the peptide observed in the CaM kinase II assay and in cultured cells, the lung was selected as the target organ since the animals would not require respiratory function until birth. The 3.7-kb 5' promoter region of the human SP-C gene dictates specific expression to type II cells of the distal airway epithelium (18). Activation of gene expression by the 3.7-kb SP-C promoter occurs
approximately on day 11 of fetal life, when a definable lung epithelium first appears.

Our transgene was composed of the SP-C promoter, the four-repeat MLCK peptide concatemer gene, and SV40 RNA processing motifs (Fig. 3a). Offspring of transgenic founder mice displayed varying degrees of lung pathology, which corresponded to congenital cystic adenomatoid malformation. In this condition, the terminal bronchioles continue to develop in the absence of alveolar epithelial proliferation. At birth, trapped air causes cystic dilation of the bronchioles. Alveoli that appeared normal were found among the cysts in some transgenic animals. One founder, a female, demonstrated no obvious physical symptoms. Her litters contained two transgenic phenotypes. Some pups displayed cyanosis and died within 15 min of birth (Fig. 3b). All of the surviving transgenic animals were females. They demonstrated retarded post-natal development as exhibited by a 10–20% reduction in body size and displayed delayed hair growth, eye opening, and tooth eruption (Fig. 3c). Affinity-purified antibodies produced against the MLCK peptide showed that expression of the transgene product occurs by approximately day 13.5 of embryonic development (Fig. 3d).

Cellular differentiation and bronchial branching began to decrease. Dilated tubules appeared at day 15.0 (Fig. 3e). There was no anti-MLCK peptide staining in the lung of day 16.5, 17.5, and post-natal male transgenic animals. Histological examination of tissue sections revealed that the transgenic male animals that died at birth had not developed an lung epithelium or lower airways (Fig. 3f). These results indicate that the type II cells that express the inhibitor peptide did not survive early development of the lung epithelium. The trachea and all major organs were histologically normal. Analysis of subsequent F1, F2, and F3 generations confirmed that all of the transgenic males died at birth and that all of the surviving transgenic animals were female (Fig. 4).

The transgene is lethal in males, suggesting an X-linked dominant trait. During morula development of females, there is random inactivation of one of the X-chromosomes, which form a Barr body. All of the cells in a given lineage inactivate the same X-chromosome throughout the life of the animal (19). In the present study the “Lyonized” females develop a mosaic lung epithelium. In some proliferating type II cells, and their clonal descendants, the CaM inhibitor peptide gene would remain silent, enabling some of the heterozygotic transgenic fe-
mammals to survive. Most of the transgenic females lived to adulthood, reproduced, and had a normal lung morphology. These studies confirm that progenitor type II cells are important for normal lung development.

Genetic manipulation of organisms at all levels of complexity has proven valuable in identifying the role of specific genes in cellular function. Gene “knock-outs” by genomic homologous recombination in embryonic stem cells allows for the selective disruption of targeted genes (20). Ablation of genes that are critical for the function of many cell types influences fetal development and can prove lethal. Indeed, disruption of fungal CaM genes demonstrates that CaM is essential for survival (21–23). Our study demonstrates that binding peptides can be used to selectively neutralize the function of a targeted protein such as CaM in a specific organelle of a defined cell type. Our findings also suggest that the mosaic inactivation of the MLCK peptide gene in lung allowed the unaffected clonal type II cells to proliferate and compensate for early loss of the cells expressing the concamper peptide. These findings demonstrate that dominant lethal genes can be sustained in the germ-line of animals by targeting transgene integration into the X-chromosome. Male progeny display the most severe phenotype, while females demonstrate a broad spectrum of phenotypes dependent upon the ratio of X-chromosome inactivation of the transgene. Some male progeny display the most severe phenotype, while females demonstrate a broad spectrum of phenotypes dependent upon the ratio of X-chromosome inactivation of the transgene.

Organism organization is based upon recognition between individual biochemical components. Cell adhesion, assembly of organelles, and signal transduction pathways are each, for example, a complex series of molecular interactions. Properties of individual proteins are altered through covalent modifications and ligand binding. Stimuli initiate a cascade of protein conformational changes. These changes, in turn, modify the properties of other proteins in sequential order. These physical interactions transmit signal bifurcation and propagation throughout the cell, ultimately leading to a cellular response. Analysis of protein-protein and protein-nucleic acid associations has shown that the interactive domains are highly structured and are often composed of less than 25 amino acids (24–30). These domains of recognition are attractive target sites for the design of peptides that modify specific cellular pathways. Identification and design of binding peptides that modify a targeted protein activity can be obtained by several techniques including biochemical fragmentation, three-dimensional structural analysis, mutagenesis of interacting species, two-hybrid transcriptional systems, and libraries of random peptides (27). Once identified, the binding properties of a selected peptide can be further refined by mutagenesis and reselection. Due to the fact that cellular processes involve a cascade of protein-protein interactions, the interference of molecular recognition by the use of binding peptides should be applicable to modulate any targeted cellular function.

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