Cdk5 regulates cell-matrix and cell-cell adhesion in lens epithelial cells

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Summary

Cdk5 is a member of the cyclin-dependent kinase family, which is expressed predominantly in terminally differentiated neurons. Lower levels of Cdk5 are also found in a wide variety of cell types, including the lens. Although Cdk5 has been shown to play an important role in neuronal migration and neurite outgrowth, its function in non-neuronal cells is not known. Therefore, this study was undertaken to explore the role of Cdk5 in the lens. Results showed that, within the adult mouse lens, Cdk5 was localized to the cytoplasm, especially along the lateral membranes of differentiating primary fiber cells, which suggests a role in cell-cell adhesion. Staining at the tips of elongating fiber cells was also particularly strong, suggesting a role in cell-matrix adhesion. To examine the possible role of Cdk5 in lens epithelial cell adhesion, we stably transfected N/N1003A rabbit lens epithelial cells with cDNAs for Cdk5 or a dominant-negative mutation, Cdk5-T33. Attachment to a fibronectin matrix, as measured with substrate-coated cell adhesion strips, was increased by Cdk5 overexpression, while an equivalent overexpression of Cdk5-T33 had no effect. Cdk5 also increased the rate of cell attachment and spreading as measured by electric cell-substrate impedance sensing (ECIS). In addition, Cdk5 overexpression decreased cell-cell adhesion as measured by a cell aggregation assay. These findings suggest that Cdk5 plays a role in regulating both cell-matrix and cell-cell interactions in the lens.

Key words: Cdk5, p35, Adhesion, Lens epithelial cells, N/N1003A cells

Introduction

Cdk5 is a serine/threonine kinase, which is primarily expressed in terminally differentiated cells, such as neurons (Lew et al., 1992; Meyerson et al., 1992; Tsai et al., 1993), muscle (Lazaro et al., 1997), and lens fibers (Gao et al., 1997). Association of Cdk5 with a regulatory subunit, such as p35, is required for kinase activation. Constitutive activity seems to be limited to neurons, which also express high levels of the Cdk5 activating proteins, p35 and p39 (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994). Many of the known substrates of Cdk5 in neurons are cytoskeletal elements or associated proteins, including neurofilament proteins, N_H and N_M (Hisanaga et al., 1995; Lew et al., 1992; Pant et al., 1997), the microtubule-associated protein, tau (Ishiguro et al., 1994; Kobayashi et al., 1995), and Pak-1 (Nikolic et al., 1998). Cdk5 is a downstream effector of Rac, a member of the Rho family of small GTPases, which regulates neuronal growth cone motility (Nikolic et al., 1998) and the cytoskeletal rearrangements involved in cell migration and ruffling (Nobes and Hall, 1995). Cdk5 activity is required for neuronal activities that depend on cytoskeletal function, such as neurite extension and proper neuronal migration during development (Nikolic et al., 1996; Ohshima et al., 1996).

A number of observations suggest that Cdk5 may also have important non-neuronal functions. Cdk5 has been implicated in differentiation of several cell types including muscle (Lazaro et al., 1997; Philpott et al., 1997), Leydig TM3 cells (Musa et al., 2000), and HL-60 cells (Chen et al., 2000). In addition, previous work from this laboratory has shown that Cdk5 and its activator, p35, are expressed in embryonic rat lens and that immunoprecipitated Cdk5 from embryonic rat lens extracts has low levels of kinase activity (Gao et al., 1997). Moreover, dominant-negative Cdk5 (Cdk5-T33) has been shown to cause abnormal lens morphology and microphthalmia in *Xenopus* embryos, suggesting that Cdk5 may regulate lens morphology and growth (Philpott et al., 1999).

The lens contains several distinct and spatially separated cell populations (Zelenka et al., 1996). The anterior surface of the lens is covered by a thin layer of epithelial cells, which undergo differentiation near the lens equator to form a posterior array of highly elongated fiber cells that makes up the bulk of the lens. Differentiating fiber cells elongate symmetrically from opposite sides of the lens, until their tips contact to form anterior and posterior sutures. In addition, differentiating fiber cells develop elaborate junctional complexes along their lateral membranes (Beebe et al., 2001). At the posterior suture, fiber cells lose their contact with the lens capsule, a specialized basement membrane that surrounds the lens. Thus, the process of fiber cell differentiation involves dynamic changes in cell-cell and cell-matrix associations. Since previous reports have indicated that Cdk5 may play a role in cell-matrix adhesion and cell migration in neurons (Ohshima et al., 1996), this study was undertaken to determine whether Cdk5 may have similar functions in the lens.

Materials and Methods

Cell culture

N/N1003A rabbit lens epithelial cells were cultured at 35°C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum

essential medium (Sigma, St Louis, MO) supplemented with 8% rabbit serum (Sigma) and 50 μg/ml gentamicin (Quality Biological, Gaithersburg, MD).

cDNA constructs and stable transfection

To make histidine-tagged Cdk5 and Cdk5-T33 constructs, *Bam*HI fragments were excised from the pCMV-Cdk5 and pCMV-Cdk5T33 vectors (Nikolic et al., 1996) and cloned into the *Bam*HI site in the pcDNA3.1/His C vector (Invitrogen, Carlsbad, CA). The cDNAs are C-terminal to the histidine tag and in reading frame. N/N1003A cells were plated on 60 mm plates at 60% confluency and transfected with 10 μg of each of the plasmid constructs using the calcium phosphate precipitation method (Ausubel et al., 1998). Cells carrying the neomycin-resistance marker were selected by addition of G418 at a concentration of 300 μg/ml after 3 days of transfection and the stably transfected cells were maintained with G418 at the same concentration.

RNA extraction and RT-PCR

For RNA extraction, 2.0×10^7 N/N1003A cells were harvested, and cytoplasmic RNA was isolated according to the manufacturer's instructions (RNAqueous™-4PCR kit; Ambion, Austin, TX). The RNA was further treated with DNase I provided in the kit, followed by a DNAase inactivation step. RT-PCR of p35 mRNA was performed according to the manufacturer's instructions (Gene Amp RNA PCR core kit; Perkin-Elmer, Boston, MA). A total of 1 μg of RNA was used with the following oligonucleotides.

Upstream: 5′-CCACCGGCCCAGCCGCCTGCACCCCCGGCC-3′ (401-430)

Downstream: 5′-GCGAGCGGTCCACGCTGCGCAGCCAGAG-CA-3′ (621-650)

The PCR protocol was 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 55°C, 1 minute at 72°C, and a final extension of 10 minutes at 72°C.

Immunohistochemistry

Two-month-old mouse eyes were embedded in paraffin and sectioned. Paraffin sections (10 μm) were placed onto silanated slides (Digene, Beltsville, MD) before immunohistochemical staining with avidin-biotinylated-peroxidase complex (ABC Kit, Vector Laboratories, Burlingame, CA). Briefly, the sections were deparaffinized by Hemo-De (Fisher, Pittsburgh, PA) twice for 5 minutes of each. After rehydration in a series of decreasing concentrations of ethanol, samples were permeablized in 0.25% Triton X-100 in PBS for 10 minutes and post-fixed in Bouin's solution (Sigma) for 15 minutes. To remove endogenous peroxidase activity, samples were incubated in PBS containing 3% hydrogen peroxide for 30 minutes. Following several washes in PBS and blocking in 5% normal goat serum in PBS, sections were incubated with either anti-CDK5 (C-8, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p35 (C-19, Santa Cruz Biotechnology) rabbit polyclonal antibodies for 1 hour. After extensive washing in PBS, secondary biotinylated antibodies (ABC kit, Vector Laboratories, Burlingame, CA) were incubated for 30 minutes. Finally, the slides were developed with Vector NovaRED and hydrogen peroxide substrate (Vector Laboratories) according to the manufacturer's instructions. Samples were then washed in distilled water, mounted with Aqua Poly mount (18606, Polysciences, Warrington, PA), and examined with a Zeiss Axioplan 2 photomicroscope. Images were captured with a CCD camera (Opelco, Sterling, VA). For controls, immunogens were included during incubation with primary antibodies.

Immunoprecipitation and immunoblotting

For immunoprecipitation experiments, cells were lysed in phosphate buffered saline (PBS), containing 1.0% Tritron X-100 (v/v), 0.5% (w/v) sodium deoxycholic acid, 1% SDS (w/v), 5 mM Na_3VO_4 (Sigma), and one Complete-Mini™ protease inhibitor cocktail tablet per 10 ml buffer (Roche, Indianapolis, IN). Pervanadate was formed by adding 30% H_2O_2 to the above extraction buffer to a final concentration of 50 mM, 10 minutes before use. Lysate containing 200 μg of protein was immunoprecipitated using anti-CDK5 mouse monoclonal IgG (DC-17; sc-249, Santa Cruz Biotechnology). For preparation of whole cell extracts, cells were lysed in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% NP-40, 20 μM Na_3VO_4 and one Complete-Mini™ protease inhibitor cocktail tablet per 10 ml buffer (Roche). Immunoblotting was performed as described previously (Gao et al., 1997) using immunoprecipitated proteins or 25 μg of cell extract. Antibodies used were as follows: rabbit polyclonal C-terminal-specific anti-Cdk5 (C-8; sc-173, Santa Cruz Biotechnology), anti-p35 rabbit polyclonal IgG (C-19; sc-820, Santa Cruz Biotechnology); mouse monoclonal anti-N-cadherin (Zymed, South San Francisco, CA). Immunoreactive bands were detected by enhanced chemiluminescence (ECL-Plus; Amersham Life Science, Piscataway, NJ) using horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology).

Cell adhesion assays

The 'stick and wash' assay was done using CytoMatrix™ Cell Adhesion Strips (Chemicon International, Temecula, CA), according to manufacturer's recommendations. Cultured cells, at 70-80% confluency, were dissociated using 2 mM EDTA, and the cell suspension was brought to a density of 5×10^5 cells/ml in PBS. Cells were applied to microplate wells at 5×10^4 cells per well and incubated for 1 hour at 37°C.

The centrifugation adhesion assay was carried out using Falcon 96-well flat-bottom polyvinyl chloride plates (BD Biosciences, Bedford, MA), following previously established protocols (McClay et al., 1981). To coat the plates with substrate, 50 μl per well of 10 μg/ml fibronectin (Gibco Invitrogen Corporation, Carlsbad, CA) was added and incubated for 30 minutes at room temperature. The plates were then rinsed with PBS to remove unbound substrate and kept on ice. Cells were added to each well (5×10^5 cells/well), and the wells were filled with tissue culture medium without rabbit serum. The plates were then centrifuged for three minutes at 35 ***g*** at 4°C in a low-speed centrifuge with a microtiter plate carrier to force the cells into contact with the substrate. The plates were incubated on ice or at 37°C for the specified lengths of time. At the end of the incubation period, the plates were inverted and subjected to a defined dislodgement shear force by centrifugation. As a negative control, cells were tested for adhesion to wells coated with BSA (40 mg/ml). All substrate-containing wells were also treated with BSA to block nonspecific binding prior to the adhesion of cells.

For both adhesion assays, adhesion was quantified as follows. Adhesion strips or microtiter plates with bound cells were gently washed to remove unattached cells. The remaining cells were stained with 0.2% crystal violet in 10% ethanol. The stain was solubilized using a 50/50 mixture of 0.1 M NaH_2PO_4 (pH 4.5) and ethanol, and absorbance was measured at 540 nm on a microplate reader.

For immunocytochemistry of vinculin and specific staining of F-actin, N/N1003A cell suspensions were prepared as described for the centrifugation assay, but cells were centrifuged onto fibronectin-coated coverslips placed within 6-well plates (instead of 96-well plates). After the plates were incubated at 4°C or at 37°C, they were rinsed with PBS and fixed with 3.7% formaldehyde for 10 minutes at room temperature. Fixed cells were rinsed with PBS containing 0.1% Triton X-100 for 5 minutes, blocked with PBS containing 1% bovine serum albumin for 1 hour at room temperature, and then incubated with a 1:100 dilution of mouse monoclonal anti-vinculin primary antibody (V-4505, Sigma), followed by rhodamine-conjugated goat

anti-mouse (115-195-146, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. For specific staining of F-actin, cells were cultured and fixed as above, then incubated with a 1:40 dilution of rhodamine phalloidin (R-415, Molecular Probes, Eugene, OR) for 20 minutes at room temperature.

Cell aggregation assay

Semi-confluent cells were seeded 1 or 2 days before use. To dissociate the cells, a solution containing 0.05% trypsin, 1 mM $CaCl_2$, and 1× PBS (calcium- and magnesium-free) was added, and the dishes were incubated at 37°C for 10-15 minutes. Cells were then collected, centrifuged for 4 minutes at 700 ***g*** at 4°C and resuspended in ice-cold PBS containing 0.05% soybean trypsin inhibitor. The cells were centrifuged again at 700 ***g*** for 4 minutes at 4°C, and resuspended in ice-cold PBS, bringing the concentration to 5×10^4 cells/ml. To 24-well tissue culture plates, precoated with BSA, 0.5 ml of the cell suspension was added, and the plates were placed on a gyrating shaker set at 60 rpm and incubated for 1 hour at 37°C in the presence or absence of 1 mM calcium. The plates were then placed on ice to prevent further cell aggregation, and 0.5 ml 8% (w/v) paraformaldehyde was added to each well. To determine the extent of cell aggregation, particle numbers were determined using a coulter counter, which was set to detect all particles (single cells as well as aggregates) greater than the threshold of 8 μm. Since calcium is required for the formation of cadherin-dependent junctions, we were able to compare the extent of calcium-dependent aggregate formation in non-transfected and transfected cells. This assay compares the total number of particles with a diameter greater than 8 μm in the absence of calcium (fully dissociated cells) to the number in the presence of calcium (cell aggregates). The average number of cells per aggregate was calculated as the ratio of these values.

Cell fractionation

For cell fractionation, cells were prepared as described for the cell aggregation assay above and allowed to aggregate in the absence or presence of calcium for 1 hour. The cells were pelleted by centrifugation at 4°C and lysed on ice with a buffer containing 300 mM sucrose, 0.5% Triton X-100, 10 mM imidazole (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 0.5 mM NaF, 1 mM Na_3VO_4 and 1 Complete-Mini^{TM} protease inhibitor cocktail tablet per 10 ml buffer, incubated on ice for 15 minutes and centrifuged at 12,000 ***g*** for 10 minutes at 4°C to pellet insoluble material. The Triton X-100 insoluble pellet was then solubilized in a solution containing 9 M urea, 4% NP-40 and 10 mM DTT, incubated at room temperature for 15 minutes and centrifuged at 12,000 ***g*** for 10 minutes. Supernatants from the two fractionation steps were immunoblotted with anti-N-cadherin antibody (Zymed) as described above.

Impedance measurements with electric cell-substrate impedance sensing (ECIS)

The impedance of cells in culture was measured using a technique described previously (Giaever and Keese, 1993; Reddy et al., 1998; Wang et al., 1995). Electrode arrays, relay bank, lock-in amplifier and software for the ECIS measurements were obtained from Applied Biophysics (Troy, NY). In this study, each electrode array consists of five wells, which are 1 cm in height and 0.5 cm^2 in area; each well contains a 250 μm diameter gold electrode and a much larger gold counter electrode. The large electrode and the small electrodes are connected via the relay bank to a phase-sensitive lock-in amplifier, and AC current is applied through a 1-MΩ resistor to the electrodes at a frequency of 4000 Hz. For impedance measurements, the electrode array was placed in an incubator and medium (200 μl/well) was added over the electrodes. After the baseline was established, three small wells were inoculated with 200 μl of cell suspensions (5×10^4 cells/well) providing nearly confluent cultures. The cells were then allowed to attach and spread for 5 hours to obtain the normalized resistance.

Results

Cdk5 and p35 localization in the lens

The localization of Cdk5 within the adult mouse lens was examined by immunofluorescence (Fig. 1). Cdk5 staining was observed primarily in epithelial cells (Fig. 1B, single arrow) and in the superficial, elongating fiber cells (Fig. 1B, double arrow; Fig. 1C), and was restricted to the cytoplasm. Staining was especially pronounced along the lateral membranes of elongating fiber cells (Fig. 1C, double arrow), and along the basal aspect of the epithelial cells (Fig. 1C, single arrow; Fig. 1D, double arrow). Staining at the posterior tips of elongating fiber cells, where they contact the lens capsule, was also particularly strong (Fig. 1E, single arrows). Cdk5 stained very weakly at the posterior suture (Fig. 1B,F, arrowhead).

A similar staining pattern was observed for p35 (Fig. 2). Staining for p35 was localized to the epithelium (Fig. 2B, single arrow) and the superficial, elongating fiber cells (Fig. 2B, double arrow). Staining was cytoplasmic (Fig. 2C) and was especially strong along the basal side of the epithelial cells (Fig. 2C, single arrow) and the lateral membranes of the fiber cells (Fig. 2C). As the elongating cells approached the posterior suture, staining for p35 decreased (Fig. 2B,D, arrowhead). The decline in p35 staining in this region was even more pronounced than that of Cdk5 (Fig. 1B,F).

Expression of Cdk5 and p35 in stably transfected N/N1003A cells

To examine the biological role of Cdk5 in the lens, N/N1003A cells were stably transfected with histidine-tagged constructs of either the catalytic subunit of Cdk5 or a dominant-negative mutation of Cdk5 (Cdk5-T33), which has no kinase activity due to a K to T substitution at amino acid 33 (Nikolic et al., 1996). The mutated protein exerts its dominant negative effect by sequestering endogenous activators with high affinity. These constructs were expressed with similar efficiency, at a level about five times greater than endogenous Cdk5 and did not affect the expression of endogenous Cdk5 (Fig. 3A). Immunoblotting of proteins from transfected and non-transfected cells demonstrated that Cdk5 overexpression also did not affect the expression of endogenous p35 (Fig. 3A).

Expression of p35 mRNA

To confirm the endogenous expression of Cdk5 activator, p35, RNA was extracted from confluent cultures of N/N1003A cells, and RT-PCR was performed using oligonucleotides specific for p35. A single RT-PCR product of the predicted size was detected (Fig. 3B), and sequencing of the RT-PCR products confirmed that it was, in fact, derived from p35.

Cdk5 promotes cell-matrix adhesion

To test whether Cdk5 plays a role in cell adhesion, we compared the adhesion properties of stably transfected and non-transfected N/N1003A lens epithelial cells using adhesion

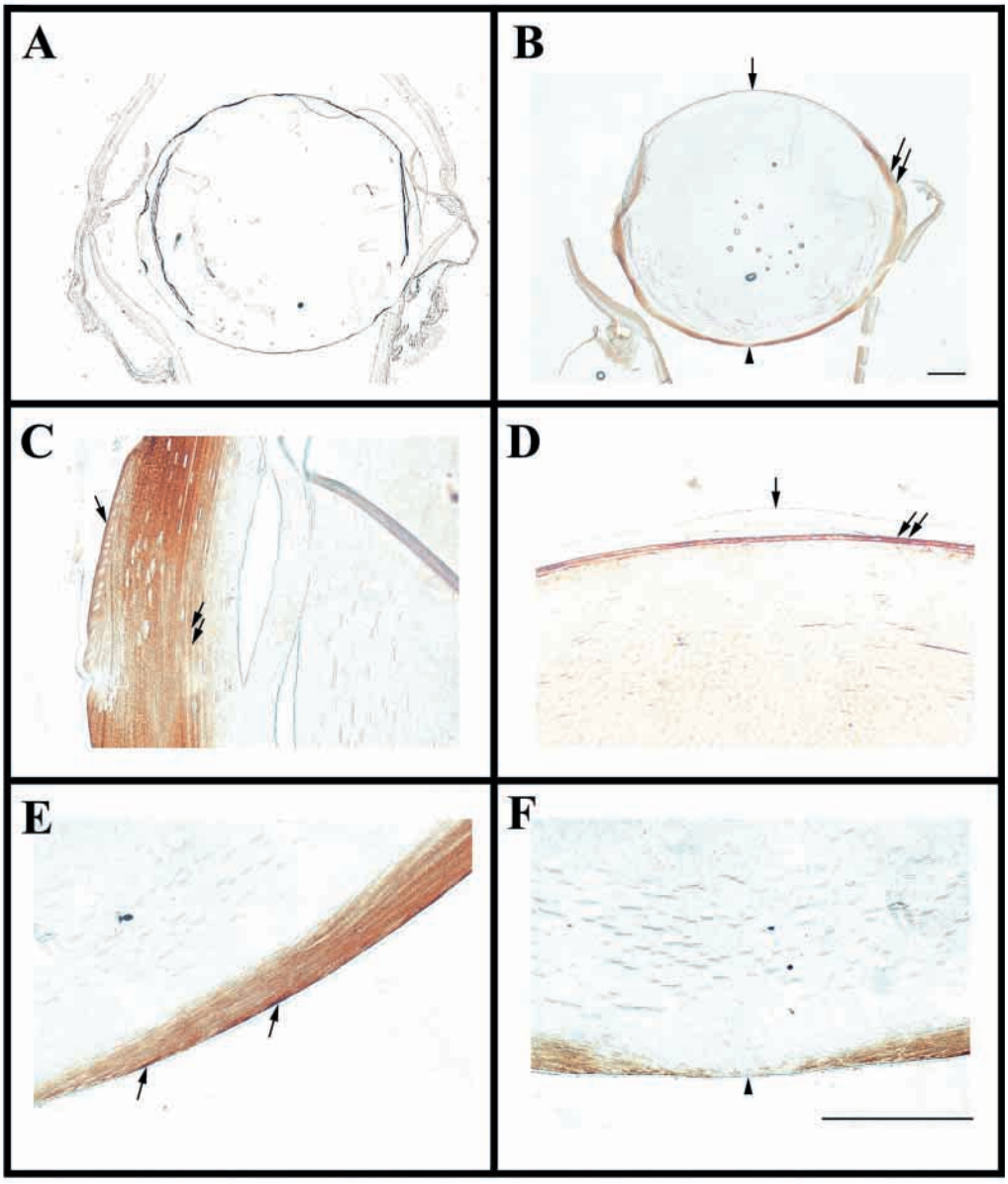

Fig. 1. Immunolocalization of Cdk5. **(**A) Control section incubated with Cdk5 antibody in the presence of blocking peptide. (B) Section incubated with Cdk5 antibody, showing positive staining in the epithelium (single arrow) and the bow region (double arrow), extending to the suture (arrowhead). (C) Higher magnification of the bow region, showing stronger staining along the basal side of the epithelial cells (single arrow) and along the membranes of fiber cells (double arrow). (D) Higher magnification of the anterior portion of the lens, showing the unstained capsule (single arrow) and strong staining in the epithelium (double arrow). (E) Higher magnification of the elongating fiber cells showing strong staining along the capsule (single arrows), where fiber cells attach. (F) Cdk5 staining is substantially decreased at the suture (arrowhead). Bar, 250 μm (A,B); 125 μm (C-F).

strips, a centrifugation adhesion assay and ECIS. To determine what substrate to use for the various assays, we tested adhesion to various extracellular matrix proteins using substrate-coated adhesion strips (Fig. 4A). Since N/N1003A cells were found to bind preferentially to fibronectin and collagen IV, fibronectin was used as a substrate in subsequent experiments. The effect of Cdk5 overexpression on adhesion to a fibronectin matrix was examined by comparing the adhesion properties of transfected and non-transfected N/N1003A cells (Fig. 4B) in the presence of serum-free medium. Cdk5 overexpression resulted in increased cell attachment to fibronectin, while Cdk5-T33 overexpression did not result in a significant effect.

Assaying cell adhesion by attachment to adhesion strips combines the effects of initial cell adhesion to substrate and subsequent strengthening steps, followed by cell spreading and the formation of focal adhesion plaques. To look at these various components of adhesion individually, we used a three step centrifugation assay (Fig. 5). The initial binding of cells to matrix was examined by incubating cells at 4°C (on ice) for 5 minutes, before centrifuging to dislodge loosely bound cells. Specific staining for F-actin and vinculin after this treatment showed that cells remained rounded with no visible organization of the actin cytoskeleton or of vinculin-containing adhesion plaques (Fig. 5A,D). The next step, which involves the recruitment of actin cytoskeleton to sites of cell attachment was examined by increasing the incubation temperature to 37°C. Following this treatment, organized cortical F-actin was visible by immunostaining, and the cells had spread slightly, although there were still no apparent adhesion plaques (Fig. 5B,E). The major component of cell spreading, the clustering of integrins and the formation of adhesion plaques was examined by incubating the cells at 37°C for 2 hours. This treatment was accompanied by formation of actin stress fibers, assembly of vinculin-containing focal adhesion plaques, and a great increase in cell area (Fig. 5C,F). Cdk5 overexpression affected all three components (Fig. 6). Cdk5 appeared to interfere slightly with both initial binding and early strengthening steps relative to the adhesion observed in the

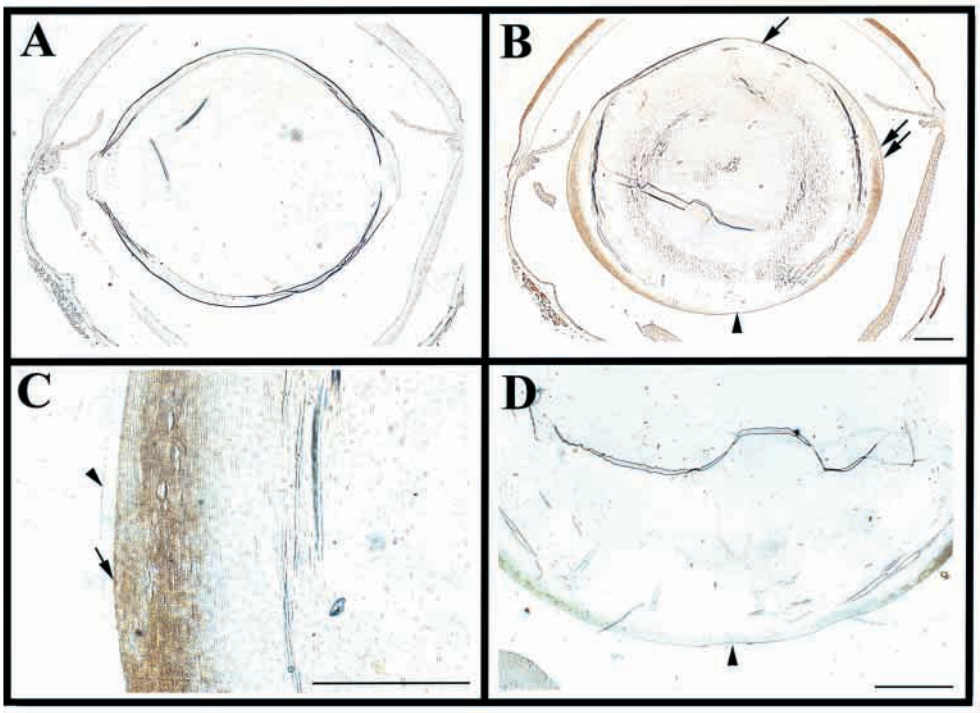

Fig. 2. Immunolocalization of p35. (A) Control section incubated with antibody in the presence of blocking peptide. (B) Section incubated with p35 antibody, showing positive staining in the epithelium (single arrow) and the superficial, elongating fiber cells (double arrow), extending to the suture (arrowhead). (C) Higher magnification of the superficial, elongating fiber cells and overlying epithelial cells, showing staining along the basal side of the epithelial cells (single arrow) and the unstained capsule (arrowhead). (D) Posterior suture, where staining is substantially decreased. Bar, 250 μm (B,D); 125 μm (C).

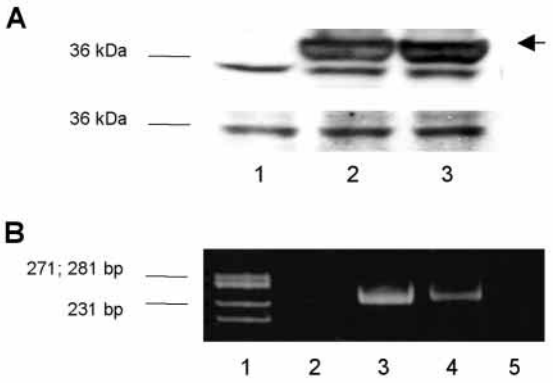

Fig. 3. (A) Protein extracted from non-transfected and stably-transfected N/N1003A cells was immunoblotted with antibody to Cdk5 or p35. Endogenous Cdk5 is expressed equally well in non-transfected N/N1003A cells (1), Cdk5-transfected N/N1003A cells (2) and Cdk5T33-tranfected N/N1003A cells (3). The arrow points to exogenous his-tagged Cdk5. (B) Expression of p35 mRNA. RT/PCR was performed on RNA extracted from rat lens (lane 3) or N/N1003A cells (lane 4). Negative controls lacking reverse transcriptase were performed with each sample: rat lens (lane 2); N/N1003A cells (lane 5). Molecular weight markers are shown in lane 1.

nontransfected or Cdk5-T33-transfected cells. However, its primary contribution was in the final stages of adhesion, as shown by the marked increase in adhesion seen in Cdk5 overexpressing cells when the incubation period at 37°C was increased from 5 minutes to 2 hours. Thus, Cdk5 overexpression seems to enhance cell-substrate adhesion by promoting cell-spreading and the assembly of adhesion plaques.

These findings are consistent with results we obtained using the ECIS technique to measure cell adhesion. In this

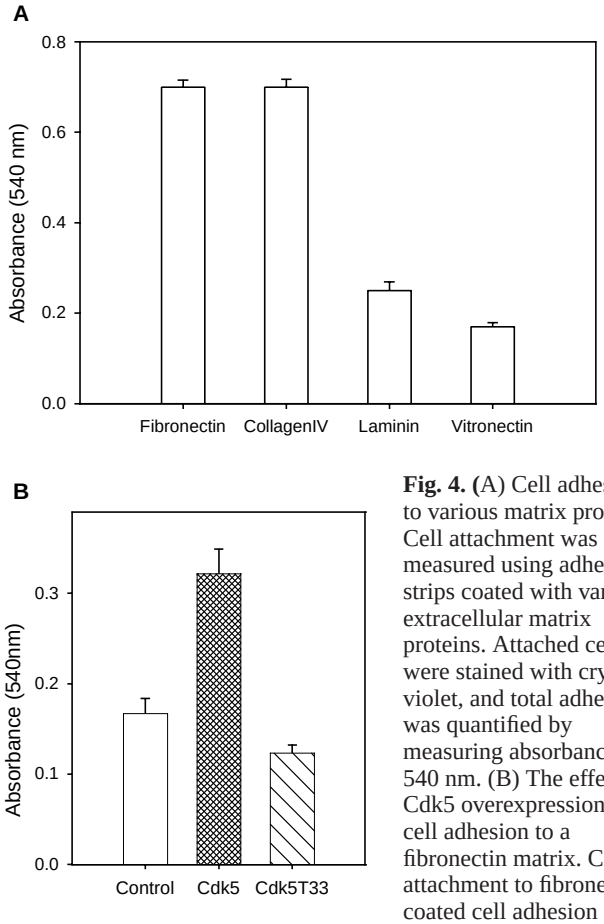

Fig. 4. (A) Cell adhesion to various matrix proteins. Cell attachment was measured using adhesion strips coated with various extracellular matrix proteins. Attached cells were stained with crystal violet, and total adhesion was quantified by measuring absorbance at 540 nm. (B) The effect of Cdk5 overexpression on cell adhesion to a fibronectin matrix. Cell attachment to fibronectin-coated cell adhesion strips was measured for cells that were stably transfected with Cdk5 or a dominant-negative construct, Cdk5T33. Attached cells were stained with crystal violet and total adhesion was quantified by measuring absorbance at 540 nm.

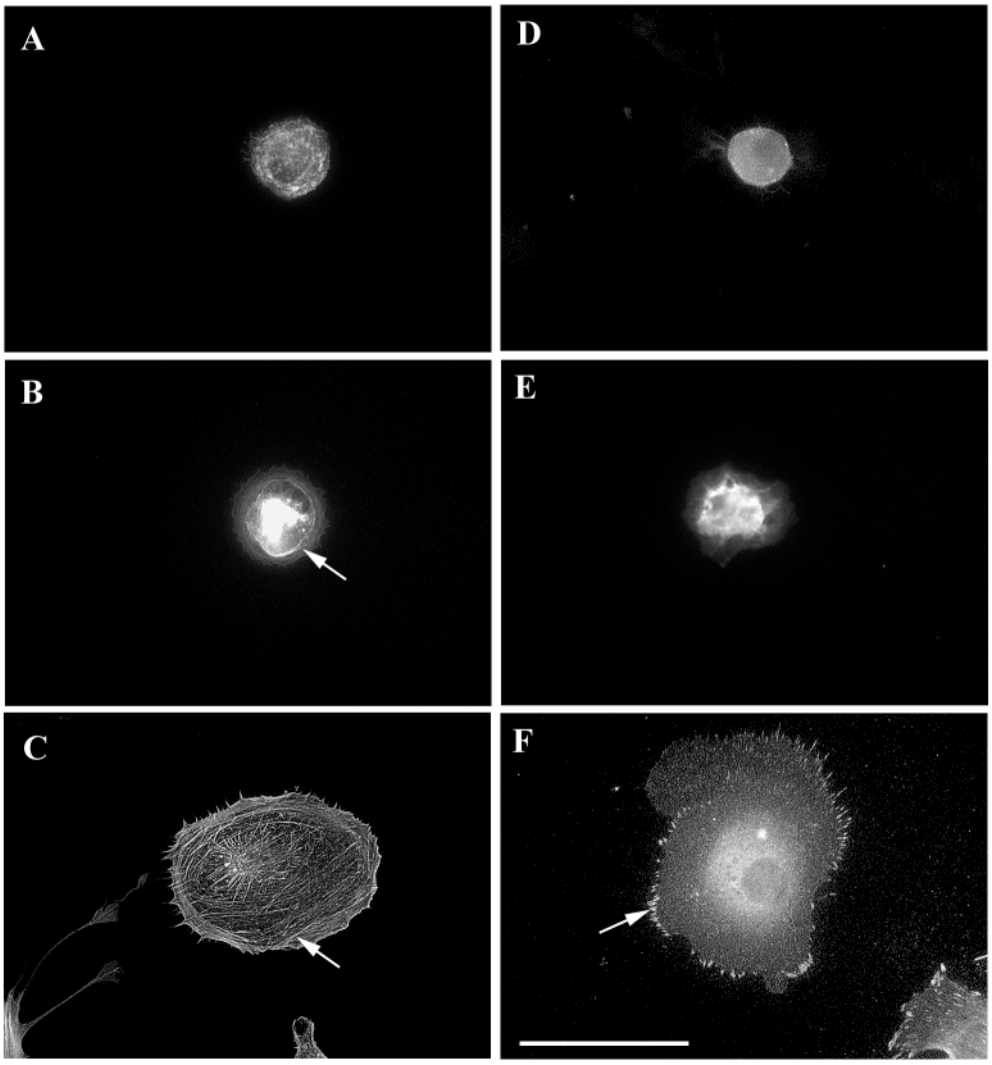

Fig. 5. Localization of actin and vinculin. Cells were centrifuged (35 ***g***) onto fibronectin-coated slides, and incubated as follows: (A,D) 5 minutes, 4°C; (B,E) 5 minutes, 37°C; (C,F) 2 hours, 37°C. Following incubation, cells were stained with phalloidin to detect F-actin (A-C) or with anti-vinculin antibody for immunocytochemical localization of vinculin (D-F).

assay, time-dependent resistance measurements were made on non-transfected and stably transfected N/N1003 cells cultured on a small gold electrode. When cells attach and spread over the electrodes, the insulating plasma membranes block the current path, leading to an increase in resistance. Cdk5 overexpression resulted in faster cell attachment as shown by the faster rate of increase in resistance; the most significant difference being in the 1-2 hour period, as previously seen using the centrifugation assay (Fig. 7). This can be interpreted as an increased rate of spreading, followed by the formation of focal adhesions. Cdk5-T33 overexpression did not result in similar changes in the time course of resistance measurements, suggesting that the observed effect depends on Cdk5 activity. Interestingly, after reaching a peak, the impedance dropped to an intermediate value, which was independent of Cdk5 expression, possibly marking a point where the cells establish strong cell-cell contacts.

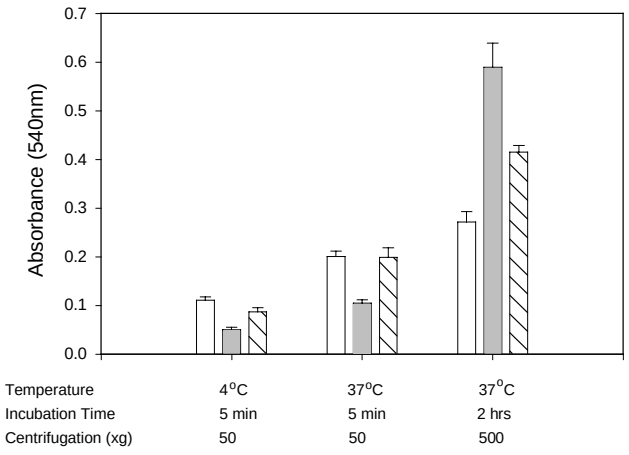

Fig. 6. Cell adhesion as measured with a centrifugation assay. Cells were centrifuged (35 ***g***) on to fibronectin-coated 96-well polyvinyl chloride (PVC) plates and incubated on ice for 5 minutes, at 37°C for 5 minutes or for 2 hours. The plates were then inverted and centrifuged at the indicated speed for 5 minutes to remove weakly bound cells. The remaining attached cells were stained with crystal violet and total adhesion was quantified by measuring absorbance at 540 nm. Open bars, nontransfected N/N1003A cells; gray bars, Cdk5-transfected cells; and hatched bars, Cdk5-T33-transfected cells. Results shown are the average of eight measurements ±s.e.

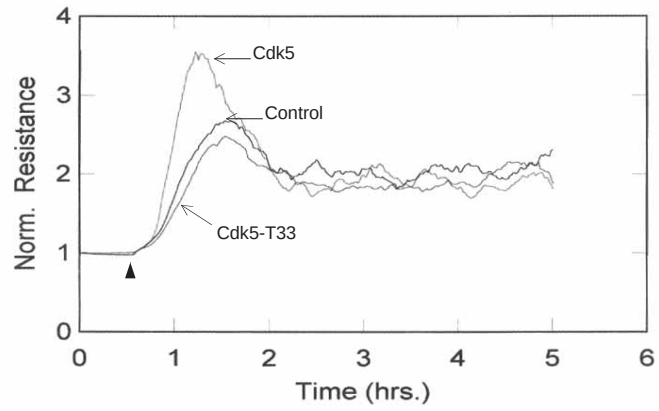

Fig. 7. Time-dependent resistance measurements on cells overexpressing Cdk5. Cultured cells were plated in chambers containing gold electrodes, and time-dependent resistance measurements were made. The resistance peak for Cdk5-transfected cells is significantly higher, and reaches a maximum at an earlier time than for non-transfected or Cdk5T33 transfected N/N1003A cells.

Cdk5 overexpression reduces cell-cell adhesion

Since Cdk5 has previously been suggested to have a role in cadherin-mediated cell-cell adhesion, we also examined the effect of Cdk5 cadherin-dependent cell-cell adhesion using an aggregation assay (Fig. 8A). Since calcium is required for the formation of cadherin-dependent junctions, this assay compares the total number of particles with a diameter greater than 8 μm in the absence of calcium (fully dissociated cells) to the number in the presence of calcium (cell aggregates). The average number of cells per aggregate can then be calculated as the ratio of these values. As expected, in the presence of calcium, the number of particles decreased (as aggregates formed) for both non-transfected and transfected cells. However, the average number of cells per aggregate was significantly smaller for cells expressing Cdk5 than for non-transfected or Cdk5-T33 expressing cells. These findings demonstrate that Cdk5 expression decreased cell-cell adhesion.

When cells were fractionated into soluble and insoluble fractions with non-ionic detergent, immunoblotting with N-cadherin antibody showed that calcium increased the amount of N-cadherin associated with the detergent-insoluble, cytoskeletal fraction in N/N1003A cells (Fig. 8B). This demonstrates that the cells form N-cadherin-dependent junctions in the presence of calcium. To determine whether the observed changes in calcium-dependent cell-cell adhesion resulted from an effect of Cdk5 on N-cadherin junction formation, we compared the amount of N-cadherin associated with the cytoskeletal fraction in nontransfected cells, Cdk5-transfected cells, and Cdk5T33-transfected cells (Fig. 8C). The results showed that Cdk5 overexpression decreased the amount of N-cadherin associated with the cytoskeleton. Thus, Cdk5 seems to reduce calcium-dependent aggregation by decreasing the formation of N-cadherin-dependent junctions.

Since overexpression of Cdk5 produced effects on cell adhesion and aggregation that were not seen in cells expressing equivalent amounts of the kinase-inactive mutation Cdk5T33, Cdk5 kinase activity seemed to be required for the biological effects. However, we were unable to measure Cdk5 kinase activity in these cells, using assays that successfully detected

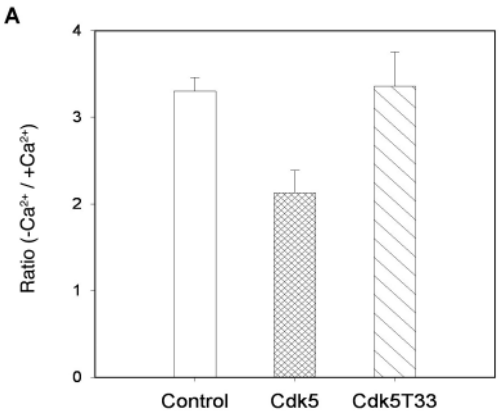

Fig. 8. The effect of Cdk5 overexpression on cell aggregation. (A) Dissociated cultured cells were incubated in the presence or absence of calcium, and the extent of cell aggregation was determined using a coulter counter threshold diameter of 8 μm. The total number of particles in the absence of calcium was divided by the number of particles formed in the presence of calcium, to calculate cell number/aggregate. (B) Immunoblot with N-cadherin antibody of the detergent insoluble fraction of N/N1003 cells incubated in the absence (lane 1) or presence (lane 2) of calcium. (C) Immunoblot with N-cadherin antibody of the detergent insoluble fraction of N/N1003 cells, Cdk5-transfected N/N1003 cells, and Cdk5T33-transfected cells incubated in the presence of calcium (top panel). The immunoblot was stained with Ponceau Red to confirm that equivalent amounts of protein were loaded. The major stained band is shown for comparison (bottom panel).

Cdk5 kinase activity in brain (results not shown). Therefore, we looked for other indications of Cdk5 activity in cells transfected with Cdk5 by immunoprecipitating Cdk5 and examining the co-immunoprecipitated p35. As shown in Fig. 9, transfection with Cdk5 increased the amount of p35 that co-immunoprecipitated with Cdk5, demonstrating that the amount of potentially active Cdk5-p35 complex is elevated by overexpression of Cdk5. Overexpression of Cdk5T33 also increased the amount of co-immunoprecipitated p35, indicating that inactive Cdk5T33/p35 complexes are present in these cells. Two bands of p35 were observed, which presumably correspond to the autophosphorylated and unphosphorylated forms (Patrick et al., 1998). In Cdk5T33-transfected cells the lower band predominated, as expected, given the inability of the Cdk5T33/p35 complex to autophosphorylate the p35 subunit. In contrast, both p35 bands

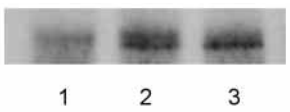

Fig. 9. Effect of Cdk5 overexpression on formation of Cdk5-p35 complexes and p35 autophosphorylation. Cdk5 was immunoprecipitated from cell lysates and the immunoprecipitated proteins were immunoblotted with anti-p35 antibody. (1) N/N1003A cells; (2) N/N1003A cells stably transfected with Cdk5; (3) N/N1003A cells stably transfected with Cdk5T33.

co-immunoprecipitated with Cdk5 in the Cdk5-transfected cells (Fig. 9A, lane 2), and both were stronger than the corresponding bands in the non-transfected cells. Thus, autophosphorylation of p35, an indicator of intracellular Cdk5 kinase activity, seems to be increased by stable transfection with Cdk5 and decreased by stable transfection with Cdk5T33.

Discussion

Like other members of the cyclin dependent kinase family, Cdk5 can be activated only when complexed with an activating protein (Ishiguro et al., 1994; Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994). Two such activators, p35 and p39, have been described in the brain (Ishiguro et al., 1994; Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994). Expression of p35 has also been reported in some non-neuronal cell types (Gao et al., 2001; Gao et al., 1997; Lazaro et al., 1997; Musa et al., 2000). Here, we show that both Cdk5 and p35 are expressed in the mouse lens and in N/N1003A rabbit lens epithelial cells. In addition, both Cdk5 and p35 were found to localize to epithelial cells and to elongating fiber cells within the lens. This pattern of expression is consistent with a role in the regulation of cell-substrate attachment and cell-cell interactions in the lens, raising the possibility that Cdk5 may be a general regulator of cell adhesion in non-neuronal cells as well as neuronal cells.

We have tested for effects of Cdk5 on cell adhesion by overexpressing the catalytic subunit Cdk5, or a kinase inactive mutation Cdk5T33, in cultured rabbit lens epithelial cells. Although we have not been able to detect significant Cdk5 activity in the transfected cells, several lines of evidence support the interpretation that overexpression of Cdk5 increases the intracellular Cdk5 kinase activity. Most importantly, we have found that equivalent overexpression of the kinase inactive form does not mimick the biological effects of Cdk5. Cdk5 and Cdk5T33 seem to be highly similar in overall structure, as shown by their common ability to interact with other proteins, such as p35 (Nikolic et al., 1996) and Cables (Zukerberg et al., 2000). Moreover, the amino acid replacement in Cdk5T33 is located in a region of the molecule that would not be expected to disrupt the tertiary structure (Jeffrey et al., 1995). Thus, Cdk5T33 is likely to mimick any effects of Cdk5 that are independent of Cdk5 kinase activity. We have also demonstrated that overexpression of Cdk5 increases the amount of Cdk5-p35 complex in the cells. This complex is active without further modification in vitro (Lew et al., 1995) and its formation is essential for increased activity in vivo. Finally, we have looked for evidence of autophosphorylation of p35 by Cdk5-p35 as a measure of intracellular Cdk5 kinase activity (Patrick et al., 1998). Immunoprecipitation of Cdk5 followed by immunoblotting for p35 showed two p35 bands, consistent with the presence of phosphorylated and unphosphorylated forms. Since the lower band predominated in Cdk5T33-transfected cells, where autophosphorylation will be inhibited by the presence of inactive Cdk5T33/p35 complexes, we consider the lower band to represent the unphosphorylated form. Cdk5-transfected cells contained both forms of p35, and both bands were significantly stronger than the corresponding bands in non-transfected cells. Thus, overexpression of Cdk5 seems to increase the autophosphorylation of p35, providing evidence that the additional Cdk5-p35 complexes formed as a result of Cdk5 transfection are enzymatically active.

The results of this study indicate that Cdk5 promotes cell-matrix interactions in N/N1003A rabbit lens epithelial cells. We find that Cdk5 has a slight inhibitory effect on the initial attachment of cells to substrate and the initial recruitment of cytoskeletal proteins to sites of cell-substrate adhesion, but strongly promotes later stages of adhesion, which involve cell spreading and focal adhesion complex formation. A role for Cdk5 in adhesion and migration is consistent with a previous report that p35 interacts with activated Rac, allowing Cdk5 to phosphorylate Pak1 (a protein kinase that regulates actin polymerization). Within the fiber cells, Cdk5 and p35 are located along the lateral membranes of differentiating fiber cells and along the capsule, primarily at the tips of elongating fiber cells. These tips consist of elaborate adhesion complexes (Bassnett et al., 1999), which mediate the attachment of elongating fiber cells to the posterior capsule.

This study also suggests a role for Cdk5 in regulating N-cadherin-dependent cell-cell adhesion in the lens. We have found that overexpression of Cdk5 leads to decreased cell-cell adhesion and reduces the association of N-cadherin with the cytoskeleton in lens epithelial cells. These findings are in agreement with a previous report showing that loss of Cdk5 activity is correlated with increased aggregation in embryonic cortical neurons and demonstrating that the Cdk5-p35 complex interacts directly with β-catenin and N-cadherin (Kwon et al., 2000). N-cadherin is the primary cadherin expressed in the lens (Volk et al., 1987; Duband et al., 1988; Beebe et al., 2001), and is required for the formation of cadherin-dependent junctions during differentiation of lens epithelial cells to lens fibers (Menko and Boettiger, 1988). Immunolocalization studies have shown that N-cadherin is concentrated along the lateral membranes of lens fiber cells (Duband et al., 1988; Beebe et al., 2001). We have found a very similar localization for Cdk5 and p35, but only in the elongating fiber cells, which still maintain contact with the lens capsule. Cdk5 and p35 seem to disappear as the cells approach the suture, as judged by the loss of immunohistochemical staining. Interestingly, previous observations suggest that the nature of the N-cadherin junctional complexes also changes when the elongation process is complete. For example, vinculin is associated with N-cadherin in the lateral membranes of fully elongated cells, but is found almost exclusively in the tips of elongating cells (Beebe et al., 2001). Moreover, the accessibility of N-cadherin to antibodies is altered once the cells complete the elongation process and detach from the capsule (Beebe et al., 2001). In view of these findings, we suggest that Cdk5 may modulate N-cadherin dependent junction formation in the elongating lens fibers, leading to the formation of dynamic cell-cell contacts and allowing the cells to move with respect to one another until full elongation is achieved. Loss of Cdk5 and p35 from the fully elongated cells would be expected to reduce cell-matrix adhesion while strengthening cell-cell adhesion. This would, in turn, promote detachment from the capsule while stabilizing the packing arrangement of the elongated lens fibers. Thus, Ckd5 may be a component of the regulatory pathway that integrates cell-cell adhesion with cell elongation during the differentiation of lens fiber cells.

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