STAT3-Mediated Signaling in the Determination of Rod Photoreceptor Cell Fate in Mouse Retina

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Purpose. The purpose of this study was to determine the intracellular pathways by which ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) negatively regulate the development of rod photoreceptors in the mouse retina.

Methods. Retina explant cultures derived from timed-pregnant CD-1 mice were used to monitor rod photoreceptor differentiation. CNTF was used to activate the signal transducer and activator of transcription (STAT)-3 and mitogen-activated protein kinase (MAPK) signal transduction pathways. Activation of STAT3 and MAPK were manipulated by using dominant-negative STAT3 recombinant adenoviruses and a specific inhibitor of MAPK, respectively. Explanted retinas were harvested at distinct time points and processed for immunohistochemistry.

Results. Blocking of the MAPK pathway by the MAPK inhibitor PD98059 did not affect normal development of rods in retina explants or the suppression of their appearance by treatment with CNTF. In contrast, activated STAT3 was necessary for suppression of the rod cell fate decision. A deficiency of the STAT3 pathway induced by a dominant negative STAT3 abolished inhibition of rod development by CNTF.

Conclusions. These results indicate that STAT3, but not MAPK, can critically regulate photoreceptor development during mouse retina development. (Invest Ophthalmol Vis Sci. 2004; 45:2407–2412) DOI:10.1167/iovs.04-0003

LINEAGE analysis of the vertebrate retina has shown that a common pool of dividing progenitor cells becomes progressively restricted in their potential for differentiation during development.1–4 For example, one late pool of progenitor cells present in perinatal mouse retina can give rise to rod photoreceptors, bipolar cells, and Müller glial cells. Extracellular factors, in collaboration with intrinsic factors, can determine retinal cell specification.5–10 Although no factor has yet been isolated that can direct a progenitor cell into a specific retinal cell fate, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) can specifically block the production of rod photoreceptors in both mice and chicks.11–14 However, the pathways used by these cytokines to transduce their signals into the cell are not understood.

Most growth factors and cytokines bind to receptors that activate protein tyrosine kinases. These kinases in turn stimulate transcription factors in the nucleus, resulting in mitogenic responses or differentiation signals.15 The signal transducer and activator of transcription (STAT) proteins were initially isolated from interferon-stimulated transcriptional complexes.16 They contain an SH2 domain and are phosphorylated directly by protein tyrosine kinases.17,18 After phosphorylation, activated STAT proteins dimerize in the cytoplasm and translocate to the nucleus where they can modulate transcription.19 Many cytokines and growth factors can activate the STAT signaling pathway, and STAT proteins have essential functions in cell growth, differentiation and survival.20–23

Cytokines of the IL-6 family, including CNTF and LIF, activate both STAT3 and MAPK through signal transduction through gp130 family common receptors.24–26 Our study provided evidence that STAT3, but not MAPK, activation plays a critical role during rod photoreceptor determination.

Materials and Methods

Reagents

Recombinant rat CNTF and LIF were purchased from R&D Systems (Minneapolis, MN); the MAPK inhibitor PD98059 from New England BioLabs (Boston, MA); anti-STAT3 (C20) polyclonal antibodies from C-terminal family Biotechnology (Santa Cruz, CA); and anti-phosphorylated STAT3 (P-Try705) and p44/p42 MAPK (P-Try185/P-Thr183) polyclonal antibodies from New England BioLabs. Ret-P1 monoclonal antibody recognizes an epitope on the N terminus of opsin.29,30 Anti-PKC monoclonal antibody was from Amersham (Arlington Heights, IL). Human STAT1 wild-type and dominant-negative mutant (Y701F) plasmids31 and human STAT3 wild-type and dominant-negative mutant form (Y705F) plasmids (Asao H, Fu XY, unpublished data, 2000) were used for releasing cDNA for viral construction.

Mouse Embryos and Retina Explant Cultures

All animal experiments were performed in accordance with NIH guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University School of Medicine. Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Boston, MA). Most of the litters were born on embryonic day (E)20, which was considered equivalent to postnatal day (PN)0. Embryos were dissected in cooled phosphate-buffered saline (PBS) for retina isolation. Whole retina was isolated from embryos or postnatal mice, respectively.10,32 Retinas were cultured individually in 1 ml basal medium without serum supplement as described previously.10 Every 2 days, half of the total medium was replaced by fresh medium. CNTF, LIF, and PD98059 were added as described later in the article. CNTF and LIF were dissolved in basal medium and PD98059 was dissolved in 0.01% dimethyl sulfoxide (DMSO).

Histology and Immunohistochemistry

Explanted retinas were fixed with 4% paraformaldehyde in PBS for at least 12 hours at 4°C. After three washes with PBS, fixed explants were least 12 hours at 4°C. After three washes with PBS, fixed explants were
dehydration through a series of graded ethanols and embedded in paraffin. All samples for one experiment were placed in the same blocks and sectioned for immunohistochemistry. A standard immunohistochemistry protocol was used for single or double staining using peroxidase-conjugated (Vector Laboratories, Burlingame, CA) or fluorochrome-conjugated (Jackson ImmunoResearch Laboratory, West Grove, PA) secondary antibodies. Stained sections were imaged with a microscope (Carl Zeiss Meditec, Thornwood, NY; or Olympus Corp. of America, Lake Success, NY) equipped with a digital camera or a confocal microscope (model 600; Bio-Rad, Hercules, CA).

Western Blot Assay
Whole-cell extracts were prepared, and Western blot assays performed as described previously. Briefly, retinal explants were suspended in a whole-cell extract buffer and frozen and thawed three times to lyse the cells. The supernatant was collected by microcentrifugation, and protein concentrations were measured using a spectrophotometer (Bio-Rad). Whole-cell extract (15 μg) was electrophoresed, blotted, and processed for chemiluminescence immunolabeling (SuperSignalR chemiluminescent substrate; Pierce, Rockford, IL).

Production of Recombinant Adenovirus and Viral Infection
The protocol for generation of recombinant adenovirus has been described elsewhere. In brief, wild-type and dominant-negative mutant forms of STAT1 and -3 with HA- or Flag-Tag were cloned to the poly-linker site of a pAdTrack-CMV shuttle vector. The constructs are bicistronic and also contain a green fluorescent protein (GFP) gene that is controlled by a separate cytomegalovirus (CMV) promoter. Thereafter, homologous recombination of these vectors was performed with adenoviral backbone vector pAdEasy-1 in BJ5183-competent cells. Linearized recombinant adenoviral vector DNA was transfected into 293 cells that were purchased from ATCC (Manassas, VA). Viruses were harvested and purified by CsCl banding as described. The final titer of the adenovirus ranged from $3.9 \times 10^{11}$ to $9.9 \times 10^{11}$ plaque-forming units (pfu)/mL. Small-volume aliquots were kept in liquid nitrogen, and fresh aliquots were used for each experiment. E18.5 explanted retina was infected with recombinant adenovirus at concentrations of $10^8$ to $10^9$ pfu/mL on the first day of the explant culture. Two days later, medium was replaced with fresh medium with or without CNTF.

**Figure 1.** (A) Colocalization of pSTAT3 with opsin and pSTAT3 with PKC in retinal explants viewed by confocal microscopy. E17.5 retina explants were incubated in basal medium or treated with CNTF for 12 hours. No overlap of pSTAT3 (green fluorescence) and opsin (red fluorescence) signals was found in outer retina part (top). When incubated in basal medium some pSTAT3 cells (green fluorescence) coexpressed PKC (red fluorescence) in the inner retina (bottom left). After CNTF treatment more coexpression was observed and this now extended into the outer retina (bottom right). (B) Costaining of pSTAT3 and Ret-P1 in vivo. Sections from PN1, -3, -5, and -7 eyes were used for labeling with anti-pSTAT3 (green fluorescence) and Ret-P1 (red fluorescence) antibodies. No double-labeled cells were detected. DIC, differential interference contrast; RPE, retina pigmental epithelium; ORL, outer retina layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Magnification: (A) ×100; (B) ×20.

**Figure 2.** A time window exists for CNTF induced inhibition of rod development. (A) Inhibition of rod differentiation after CNTF withdrawal was observed in cultured explants from E12.5 retina. Individually, after 0 to 12 hours and 3, 6, 9, and 12 days of CNTF treatment, the retinas were rinsed with PBS three times and fresh medium applied. After further culture, Ret-P1 (red fluorescence) was used as an opsin marker (top). Bottom: differential interference contrast (DIC) images. (B) CNTF was added to cultures of E15.5 retina at the indicated times and maintained for the remainder of the 12-day period. Ret-P1 (red fluorescence) was used as opsin marker (top). Bottom: DIC images. (C) A line chart summarizing CNTF inhibition of rod formation between E17.5 and PN2. Magnification, ×10.
RESULTS

STAT3 Expression and Activation in Retina Explants

STAT3 expression in the retina increases during late embryonic stages and declines during the first postnatal week. To test whether STAT3 activation is associated with different cell types during retina explant development, we examined the colocalization of pSTAT3 and opsin, a marker for rod photoreceptors, or PKC, a marker for rod bipolar cells. In explants from E17.5 retina, no colocalization of pSTAT3 and opsin was observed. After CNTF treatment no cells were labeled with RET-P1 but there was an increase in the pSTAT3 signal (Fig. 1A, top). We also examined the sections from the same explanted retinas, using antibodies to the bipolar cell marker PKC. Activated STAT3 protein colocalized with PKC in the inner nuclear layer when basal medium was used, and there were no PKC- and pSTAT3-positive cells in the photoreceptor layer (Fig. 1A, bottom). However, with CNTF treatment, PKC-positive cells were also found throughout the outer layer of the explant and were colocalized with activated Stat3 (Fig. 1A, bottom).

We also labeled retinal sections with anti-pSTAT3 and Ret-P1 antibodies to test whether activated STAT3 and opsin were colocalized within rod photoreceptor cells in vivo. As shown in Figure 1B, there was no colocalization of these two molecules in retinas from animals examined at PN1 to PN7. pSTAT3-positive cells were seen throughout the whole layer in the PN1 retina, with a decreasing number of positive cells over the next week, in agreement with the Western blot analysis described earlier. As the number of pSTAT3 positive cells decreased, the number of Ret-P1 positive cells increased, as seen clearly in the PN3 outer retina layer where pSTAT3 is no longer detected. This result suggests that reduction of STAT3 levels or loss of STAT3 activation in the outer retina layer may play a crucial role in initiation of rod development in vivo.

A Temporal Window for CNTF-Induced Rod Inhibition

Cells in retinal explants are capable of division, commitment and differentiation into mature neuronal cells similar to that in vivo. We used such cultures to determine the temporal sensitivity of the inhibition of rod photoreceptor formation by CNTF. Two experiments were performed. First, E12.5 embryonic retinas were dissected and treated with CNTF for a distinct period of 0 to 12 hours, or 0 to 3, 6, 9, or 12 days, and then the retina was rinsed with PBS and incubated in fresh basal medium. After culture for 2 weeks, each retina was analyzed using Ret-P1 (Fig. 2A). This experiment revealed that a continuous treatment with CNTF for 6 days (equivalent to E17.5 in vivo) was necessary to inhibit completely the appearance of Ret-P1-opsin positive cells (Fig. 2A), suggesting that some cells in the developing tissue had not gained competence to respond to the cytokine until this stage.

Second, in a complementary experiment, explants from E15.5 embryonic retinas were cultured in basal media for a period in the absence of CNTF and then treated with CNTF.
receptor fate. From E17.5 to PN2 that is critical for determination of photoreceptor production was detected (Fig. 2B). Thus, beginning with added CNTF for days 9 to 12, no inhibition of Ret-P1 labeling with the anti-opsin antibody Ret-P1. Infection with Ad-STAT3Y705F, however, totally blocked the action of CNTF, suggesting that the cytokine action requires STAT3 phosphorylation and dimerization (Fig. 4A). Recombinant adenoviruses containing wild-type STAT1 (Ad-STAT1) and a dominant-negative STAT1 (Ad-STAT1Y701F) were used as additional controls and had no effect on the inhibition of rod differentiation by CNTF (Fig. 4B). Thus, CNTF-induced inhibition of rod photoreceptor differentiation was specifically blocked by expression of dominant negative mutant of STAT3Y705F, suggesting that STAT3 signaling plays a critical role in CNTF-induced effect.

To test whether MAPK was also involved in the cytokine-induced suppression of rod photoreceptor differentiation, we used a specific inhibitor of MAPK activation, PD98059.38 E17.5 retinas were cultured for 12 days in basal medium or medium with 20 or 100 μM PD98059 (Fig. 5). The appearance of Ret-P1–positive rod photoreceptors in the retina was not affected by the presence of 100 μM PD98059, even though MAPK activation was completely inhibited at this concentration (Fig. 5D). Furthermore, inhibition of MAPK had no effect on the CNTF-induced suppression of rod formation (Figs. 5F, 5G). These results demonstrate that MAPK activation is neither required for photoreceptor differentiation nor has a role in CNTF-mediated suppression of rod photoreceptor formation.

**DISCUSSION**

The mammalian retina has been used extensively for studies of the cellular and molecular mechanisms controlling central nervous system (CNS) development. Its restricted complement of basic cell types, laminar organization, and accessibility make it

**For example**, CNTF was added from the first day of the culture until day 12 (0–12 days), or from the third day of the culture until day 12 (3–12 days), and so forth, as shown in Figure 2B. When the E15.5 mouse retinas were cultured first without CNTF for 8 days (equivalent to P2.5 given a 20-day gestation), then with added CNTF for days 9 to 12, no inhibition of Ret-P1 photoreceptor production was detected (Fig. 2B). Thus, beginning at the equivalent of PN2, the retinal tissue loses competence to respond to CNTF. In summary (Fig. 2C), the results of the two experiments suggest that there is a temporal window from E17.5 to PN2 that is critical for determination of photoreceptor fate.

**Inhibition of Rod Determination**

To determine whether either STAT3 or MAPK was responsible for the cytokine-induced suppression of rod photoreceptor differentiation, we performed the following experiments. First, we infected E18.5 retinal explants with adenoviruses containing wild-type STAT3 (Ad-STAT3), a dominant-negative STAT3 (Ad-STAT3Y705F), or vector control (Ad-GFP; Fig. 3A). Infection with Ad-GFP allowed continued expression of basal levels of STAT3 for 7 days whereas infection with Ad-STAT3 or Ad-STAT3Y705F caused overexpression of STAT3 (Fig. 3B). Monitoring of introduced STAT proteins was aided by the FLAG tag on the proteins as shown by the colocalization of anti-FLAG immunolabeling and GFP shown in the section from Ad-STAT3 infected retina explants (Fig. 3C).

Infected retinas were treated with CNTF or left untreated and the effects on rod photoreceptor production measured by labeling with the anti-opsin antibody Ret-P1. Infection with Ad-STAT3 had no effect on the ability of CNTF to inhibit rod formation (Fig. 4A). Infection with Ad-STAT3Y705F, however, totally blocked the action of CNTF, suggesting that the cytokine action requires STAT3 phosphorylation and dimerization (Fig. 4A). Recombinant adenoviruses containing wild-type STAT1 (Ad-STAT1) and a dominant-negative STAT1 (Ad-STAT1Y701F) were used as additional controls and had no effect on the inhibition of rod differentiation by CNTF (Fig. 4B). Thus, CNTF-induced inhibition of rod photoreceptor differentiation was specifically blocked by expression of dominant negative mutant of STAT3Y705F, suggesting that STAT3 signaling plays a critical role in CNTF-induced effect.

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**FIGURE 4.** STAT3 is critical for inhibition of rod photoreceptor development by CNTF (A). After 4 days of CNTF treatment, no RET-P1 signals (red fluorescence) were observed in the Ad-STAT3–infected retina compared with the explants without CNTF. Green fluorescence clearly identified infected cells in the cultured explants. RET-P1 signals (red fluorescence) were observed in Ad-STAT3Y705F–infected retina either with CNTF or without CNTF treatment. Green fluorescence from STAT3-Y705F viral infection was clearly detected in the cultured explants and overlapped with RET-P1 signals (red) at the explant surface. (B) Infection with Ad-STAT1 or Ad-STAT1Y701F did not alter rod photoreceptor development. INL: inner nuclear layer; PR, photoreceptors. Magnification, ×100.

**FIGURE 5.** MAPK does not regulate rod photoreceptor formation. Images of double labeling with Ret-P1 (red fluorescence, A–G) and anti-pp44 (green fluorescence, H–N) are shown. Explanted retinas were cultured for 12 days from E17.5, treated with PD98059 dissolved in 0.01% DMSO (B–D, I–K). No changes of Ret-P1 signals were observed, even though MAPK was inhibited. BM, basal medium; DMSO (0.01%); PD (20 and 100), PD98059 20 and 100 μM. Explanted retinas were also cultured for 12 days from E17.5 and treated with the same concentrations of PD98059 in the presence of CNTF (25 ng/mL, E–G, L–N). CNTF inhibited rod photoreceptor formation even when MAPK was inhibited. (O–U) Differential interference contrast (DIC) images. Magnification, ×40.
an excellent model system for development studies. Although much is known about the timing of the generation of retinal cell types, their lineage relationships and extracellular factors that can influence cell formation, little information is available on the signaling pathways that regulate cell fate or the downstream cascades they initiate. In this study, we have shown that STAT3, but not the MAPK, signaling pathway plays an essential role in the determination of rod photoreceptor fate in mice. Although we cannot yet prove that CNTF or LIF are the natural regulators of STAT3 in developing retina, both have been detected in retinal tissue and could serve this function. 

STAT3 expression occurred in a wave across the layers of the retina during development. At E17, STAT3 was expressed only in the inner retina but by neonatal stages is located specifically in the outer retina. Thus, STAT3 expression shows an appropriate temporal and spatial localization in the retina to play a role in rod photoreceptor formation. It is possible that STAT3 acts in similar ways to regulate development of other retinal cell types in the inner retina at earlier stages of development.

Schulz-Key et al. found that CNTF only has a role as a transient and reversible negative regulator of rod differentiation in a slice culture system from rat retina. In the continuous presence of CNTF the number of opsin-positive cells increased at a normal rate but with a delay of 3 to 4 days. This phenomenon was not observed in our system as shown in Figure 2. A major experimental difference between these experiments and those presented here is that Schulz-Key et al. used 10% horse serum in the medium. Serum contains a number of neurotrophic factors, including pigment epithelium-derived factor (PEDF), a factor known to modulate rod photoreceptor maturation. 

Whether rod photoreceptor formation is a default pathway or requires positive induction is not known. Because rod formation occurs readily in explant cultures, any inductive signals are likely to be intrinsic to the retina. Rod formation does not occur in low-density cultures of embryonic rodent retina, suggesting the need for cell interactions. Adding cells of different ages to retinal cultures can influence the rate or extent of rod photoreceptor formation and this influence is effective through membrane filters or agarose, suggesting the actions of a soluble factor. 

We propose that, in addition to natural stimulatory factors, the STAT3 signal transduction pathway acts as a brake to regulate rod photoreceptor formation. Because we did not detect STAT3 and opsin in the same cells, loss of STAT3 expression appears to be linked to rod differentiation, and the actions of STAT3 are probably occurring in a rod precursor cell or some earlier progenitor. The increase in PKC positive cells seen after CNTF treatment suggests that activation of STAT3 influence cell fate decisions, either directly or by maintaining cells in a state where they can be acted on by various positive inductive factors.

In contrast to vertebrates, we found that MAPK is not necessary for rod photoreceptor differentiation. In Drosophila, DER, a receptor tyrosine kinase, mediates signaling from Spitz to Basket through the RAS/RAF-MAPK pathway to control photoreceptor differentiation. A cytokine receptor has recently been identified in Drosophila, termed domeless or mom, that shows similarity with the IL-6 receptor family, LIFR and CNTFR. This suggests that CNTF and LIF signals can trigger pathways, even in Drosophila.

Recent studies showed that Drosophila PIAS gene (dpias), a homologue of mammalian protein inhibitors of activated STATs play a dramatic role in blood cell and eye development. The increased expression of dpias or coupling with heterozygosity for the stat92E LOF allele led to transformation events, with antennae frequently replacing eyes, and suggested that DPIAS and STAT92E naturally interact in eye formation and eye determination. It is also interesting that STAT3 homologues have been discovered in eyes of Drosophila, zebralist, and Xenopus (Zhang J, Fu XY, unpublished data, 1995), possibly implicating STAT3 as a functional molecule in eye development and function during evolution.

A central question of developmental biology has been whether the cues that guide the embryo are set up by extrinsic and/or intrinsic information. In neuronal development, signal transduction pathways activated by extracellular factors, such as PI-3K, Notch, Hedgehog, and Wnt have all been shown to control cell fate decisions at different developmental stages. Our results now indicate that STAT3 also is an essential intracellular messenger for determination of cell fate in mouse retina.

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