Growth Arrest-Specific Gene 6 (Gas6)/Adhesion Related Kinase (Ark) Signaling Promotes Gonadotropin-Releasing Hormone Neuronal Survival via Extracellular Signal-Regulated Kinase (ERK) and Akt

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We identified Ark, the mouse homolog of the receptor tyrosine kinase Axl (Ufo, Tyro7), in a screen for novel factors involved in GnRH neuronal migration by using differential-display PCR on cell lines derived at two windows during GnRH neuronal development. Ark is expressed in Gn10 GnRH cells, developed from a tumor in the olfactory area when GnRH neurons are migrating, but not in GT1–7 cells, derived from a tumor in the forebrain when GnRH neurons are postmigratory. Since Ark (Axl) signaling protects from programmed cell death in fibroblasts, we hypothesized that it may play an antiapoptotic role in GnRH neurons. Gn10 (Ark positive) GnRH cells were more resistant to serum withdrawal-induced apoptosis than GT1–7 (Ark negative) cells, and this effect was augmented with the addition of Gas6, the Ark (Axl) ligand. Gas6/Ark stimulated the extracellular signal-regulated kinase, ERK, and the serine-threonine kinase, Akt, a downstream component of the phosphoinositide 3-kinase (PI3-K) pathway. To determine whether ERK or Akt activation is required for the antiapoptotic effects of Gas6/Ark in GnRH neurons, cells were serum starved in the absence or presence of Gas6, with or without inhibitors of ERK and PI3-K signaling cascades. Gas6 rescued Gn10 cells from apoptosis, and this effect was blocked by coincubation of the cells with the mitogen-activated protein/ERK kinase (MEK) inhibitor, PD98059, or wortmannin (but not rapamycin). These data support an important role for Gas6/Ark signaling via the ERK and PI3-K (via Akt) pathways in the protection of GnRH neurons from programmed cell death across neuronal migration. (Molecular Endocrinology 13: 191–201, 1999)

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INTRODUCTION

GnRH Neurons: A Model of Tightly Controlled Neuronal Migration across Embryogenesis

GnRH is the hypothalamic releasing hormone that controls pituitary gonadotropin subunit biosynthesis and, ultimately, reproductive function (1). The GnRH neuronal population is unique, in that 800-1000 neurons migrate from the olfactory placode to the forebrain across embryogenesis in the rodent (2–5). The neurons migrate adjacent to olfactory neurons, but then diverge to reach their final destination in the hypothalamus. GnRH neurons innervate the anterior

pituitary and deliver an episodic pattern of hormone signaling to the gonadotropes that ultimately leads to normal reproductive function (1). Failure of this targeted GnRH neuronal migration results in failure of sexual maturation and GnRH deficiency syndromes (6–7). In the human, the X-linked form of GnRH deficiency has been found to result from a defect in the KAL gene, which encodes a neural cell adhesion molecule expressed in cells adjacent to GnRH neurons (8–10). The molecular defects for other forms of GnRH deficiency are unknown due, in part, to the lack of information concerning the factors that control GnRH neuronal migration.

GnRH Neuronal Cell Lines: Models to Study GnRH Expression

Our ability to directly study GnRH gene expression was advanced by the development of GnRH-producing cell lines. Mellon and colleagues (11) used the rat GnRH promoter fused to the SV_{40} T antigen in transgenic animals to target immortalization of the GnRH neuronal population. One animal developed a tumor in the forebrain, a time when GnRH neurons are postmigratory. The GT cell lines were derived from that tumor and produce large amounts of GnRH mRNA and protein (12). Similarly, Radovick and co-workers (13) used the human GnRH promoter fused to the SV_{40} T antigen in transgenic mice. An animal developed a GnRH neuronal tumor in the olfactory area, a locus for migrating GnRH neurons. The resultant Gn10, Gn11, and NLT cell lines express low levels of GnRH mRNA and protein (Ref. 13, and M. E. Wierman, unpublished observations). Based on the divergent phenotypes of the two neuronal cell lines, we hypothesized that by using the technique of differential display PCR, we could identify novel factors involved in GnRH neuronal migration and/or gene expression (14). We reasoned that GT1–7 cells would express factors resulting in high level GnRH expression and a postmigratory status. Conversely, Gn10 cells would express factors involved in GnRH neuronal migration and factors that repress GnRH expression (or lack activators of gene expression). Gn8–01 is a cDNA clone characterized in this screen from Gn10 cells and was identified as Ark, a membrane receptor tyrosine kinase that may play a role in GnRH neuronal migration, gene expression, and protection from apoptosis.

Ark (Axl): Role in Protection from Apoptosis

Ark is a mouse protein identified during a screen for homologs of the BEK fibroblast growth factor receptor (15). Ark and its human homolog, Axl, Ufo, or Tyro7 (16–18), are members of a new family of receptor tyrosine kinases that includes Tyro3 (with many alternative names) (17, 19) and Mer (20). This family is unique in that the N-terminal, extracellular portion of the molecule contains two Ig-like repeats and two fibronectin type III repeats (15–20). This combination of structural elements has been classically observed in cell adhesion molecules or receptor tyrosine phosphatases, but not in receptor tyrosine kinases. Although initially isolated as candidate growth factor receptors, members of the Ark (Axl) family are not mitogenic unless they are overexpressed at high levels in tumor cell lines (21–22). Some have suggested the importance of the extracellular domain of Ark in cell-cell adhesion due to its ability to induce homophilic binding independent of ligand in fibroblasts (21), although others have not shown an effect independent of ligand using Axl expressing 32D cells (23). The nuclear transcription factors and gene targets downstream in the Ark-signaling pathway have not been identified.

Gas6/Ark Signaling in Other Systems Protects from Programmed Cell Death

Recently, the ligand for Ark (Axl) and Tyro3 was identified as Gas6, a gene induced after growth arrest of fibroblast cells in culture (24–26). Gas6 is a soluble, vitamin K-dependent protein with homology to protein S (24–26). Studies have suggested the importance of Gas6/Axl-induced chemotaxis of 32D and vascular smooth muscle cells (23, 27). Additional studies show that Gas6/Ark or Axl signaling reduces the rate of programmed cell death in fibroblasts (28–29). We were intrigued by the identification of Ark in Gn10 GnRH neuronal cells, which were derived from migrating GnRH neurons, and hypothesized that Ark (Axl) may play a role in protection of the GnRH neuronal population from apoptosis during their migration into the forebrain. Thus, we designed experiments in the two divergent GnRH-producing cell lines to address the role of Gas6/Ark (Axl) signaling during neuronal cell death and the pathways that are activated in this process.

Pathways Involved in Neuronal Apoptosis

The downstream signaling cascades involved in apoptosis are a focus of active investigation. Many of the pathways are similar among cell types, but some cell-specific pathways have been identified. Studies have shown the importance of the extracellular signal-regulated kinase (ERK) pathway in rescue from cell death (30–35). It is unclear however, whether the activation of ERK is to trigger mitogenesis and thereby indirectly regulate apoptosis or to act directly in this process (28). Studies have also shown the importance of Akt [also called protein kinase B (PKB) and related to A and C protein kinase (RAC-PK)], a downstream component of the phosphoinositide 3-kinase (PI3-K) pathway, in the protection of cells from apoptosis (36–41). Recently, investigators have demonstrated that Akt phosphorylates the BCL-2 member, BAD, deactivating its proapoptotic actions (42). In addition to antiapoptotic signaling cascades, stress-activated proapoptotic pathways converge in activation of p38 mitogen-activated protein (MAP) kinase in neuronal cells (43).

Inhibitors of p38 have been shown to protect from various triggers of programmed cell death (43). With this background, we examined which of these pathways may be involved in Gas6/Ark signaling in GnRH neuronal cells.

RESULTS

The Ark Tyrosine Kinase Receptor mRNA and Protein Are Expressed in Gn10 and Not GT1–7 Neuronal Cells

To confirm that Ark mRNA and functional Ark protein were expressed in GnRH neuronal cells from which the clone Gn8–01 was isolated, Northern and Western blots from each neuronal cell line were performed. The full-length mouse Ark cDNA was used to probe the Northern (15). An Ark-specific antisera, no. 318, which recognizes the Ark extracellular domain (21), was used to detect the Ark protein. Figure 1 shows the presence of the Ark 3.4-kb mRNA and the 120–140 kDa protein doublet in the Gn10, but not in the GT1–7 neuronal cells. [Additional studies showed that neither neuronal cell line contains detectable levels of Gas6 mRNA or protein (data not shown).]

Ark-Positive Gn10 Neurons Are More Resistant to Growth Factor Withdrawal- Induced Apoptosis than Ark-Negative GT1–7 Cells

Prior experiments in fibroblast cell lines demonstrated that Gas6/Ark (Axl) signaling is important in protecting or rescuing cells from programmed cell death induced by serum withdrawal (28, 29). This finding prompted us to determine whether a similar function is important in GnRH neuronal cells. If Ark expression and activation protect neurons from apoptosis, then the Gn10 (Ark positive) cells might be more resistant than GT1–7 (Ark negative) neurons to growth factor withdrawal, a standard paradigm to trigger apoptosis. To test this hypothesis, the GT1–7 and Gn10 cells were grown under normal conditions and then changed to serum-free media. Various times after serum withdrawal (2–72 h), cells were stained for condensed, hyperchromatic nuclei using the Hoescht stain (33258). Gn10 GnRH neurons were less sensitive to serum withdrawal than GT1–7 cells. At baseline, they exhibited a low rate of apoptosis (2.2%) that did not increase over the first 24 h and then gradually increased to 7.3% and 12.4% at 48 and 72 h, respectively. In contrast, GT1–7 GnRH neurons were more sensitive to growth factor removal. They showed a basal level of apoptosis of 2.9%. This increased to 13.8%, 42.3%, and 41.2% at 24 h, 36 h, and 48 h, respectively. When serum was removed, both neuronal cells lost their ability to remain attached to the tissue culture dishes, which was partially prevented by culturing on polylysine-coated slides.

To further map the time course of sensitivity to serum withdrawal, both neuronal cells were placed in serum-free conditions, and cells were stained at various time intervals with acridine orange/ethidium bromide to detect apoptotic nuclei as orange-red condensed fragments (Fig. 2). In this assay, cells are lifted, thereby capturing both attached and detached cells. In GT1–7 cells, the basal level of apoptosis in serum was 2.1% and was similar at 3% at 8 h after serum withdrawal, but then increased to 28%, 35%, and 38% at 24 h, 48 h, and 72 h. In contrast in Gn10 neuronal cells, the basal rate of apoptosis was 2% and was stable over the first 24 h of serum withdrawal (2.1% and 2.5%). The levels of apoptosis then increased gradually over the next 2 days to 10.6% and 13% at 48 and 72 h (Fig. 2). Thus, there was a clear dichotomy between Ark-positive Gn10 and Ark-negative GT1–7 cells in apoptotic rates after serum deprivation. We hypothesize that

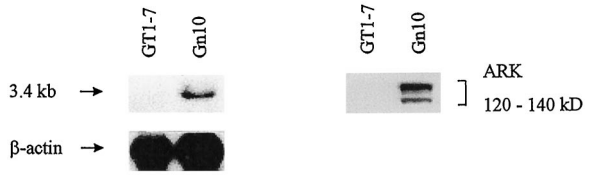

Fig. 1. Ark Is Expressed in Gn10 and Not GT1–7 Neuronal Cells

Twenty micrograms of total RNA from each neuronal cell line were separated by electrophoresis on a 1.4% agarose gel, transferred to nitrocellulose, and hybridized with a radiolabeled Ark cDNA. The 3.4-kb mRNA is Ark. Total cell lysates of the two neuronal cells were separated by electrophoresis on a 10% SDS-PAGE gel, transferred to PVDF, and probed with the No. 318 Ark antisera. The *arrow* indicates the 120- to 140-kDa Ark protein.

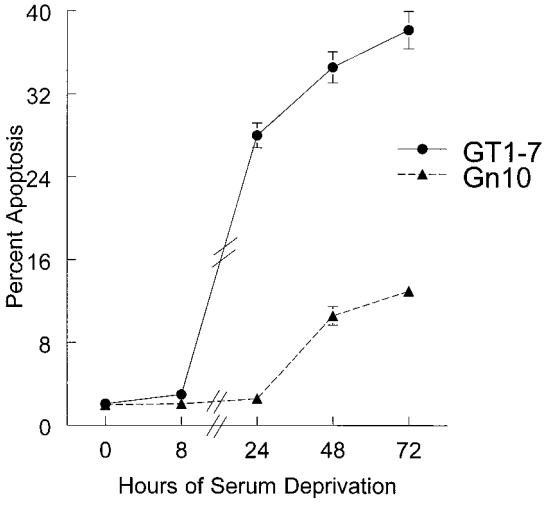

Fig. 2. Gn10 Cells Are More Resistant to Serum Deprivation-Induced Programmed Cell Death Than GT1–7 GnRH Neuronal Cells

Numbers of apoptotic cells were determined by ethidium bromide/acridine orange staining at various times after cells were placed in serum-free media.

Ark is one of many factors contributing to the differences between the two GnRH neuronal cell lines that may play a role in the regulation of programmed cell death in GnRH neurons.

Gas6/Ark Activation Triggers Multiple Signaling Cascades in GnRH Neuronal Cells

To explore the intracellular pathways by which Gas6/Ark might modulate the sensitivity to apoptosis in GnRH neurons, we asked whether rhGas6 triggered changes in the activation of ERK and Akt. ERK1 and ERK2 are components of the MAP kinase pathway known to be critical in mitogenic as well as antiapoptotic signaling in certain cell systems (30–31, 44). Akt is a downstream component of the PI3-K pathway, known to be activated by growth factors and to protect cells from apoptotic stimuli (30, 36, 38–41, 45). Phosphorylation of these proteins, as detected by phospho antisera, is correlated with their activation. GT1–7 and Gn10 cells were grown in the presence or absence of serum (48 h), and Gn10 cells were stimulated with rhGas6, 400 ng/ml, for various periods of time. Whole-cell lysates were analyzed by Western blot and probed with antisera specific for phospho-ERK and ERK (Fig. 3A) or phospho-Akt and Akt (Fig. 3B). Of interest, GT1–7, Ark-negative, cells grown in serum had lower levels of activated ERK and Akt than Gn10, Ark-containing neurons, and levels did not change with serum withdrawal (lanes 1 and 2 *vs.* lanes 3 and 4, panels A and B). However, in the presence of serum, Gn10 neuronal cells have relatively high levels of active ERK and Akt (lanes 3 and 5, panels A and B).

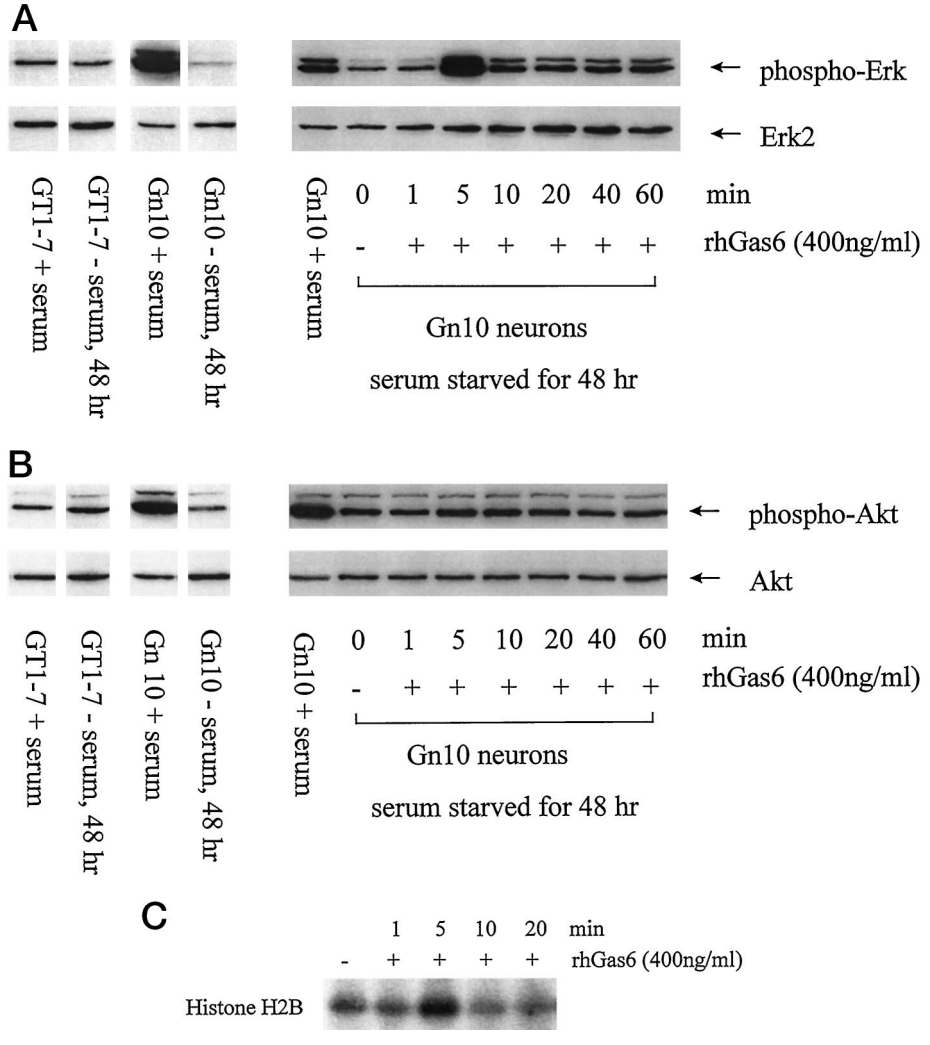

Fig. 3. Gas6/Ark Activation Is Associated with Activation of ERK and Akt

GT1–7 and Gn10 cells were grown in serum or serum starved for 48 h (*left*), and Gn10 cells were then treated with vehicle or rhGas6 (400 ng/ml) for 1–60 min (*right*). Cell lysates were separated by electrophoresis, transferred to PVDF, and incubated with antisera specific for phospho-ERK, ERK (panel A), or phospho-Akt and Akt (panel B). Results are shown from a representative experiment from three to five performed. Panel C shows phosphorylation of histone H2B by Akt in response to rhGas6 (400 ng/ml) for 1–20 min.

Serum withdrawal decreased levels of phospho-ERK and Akt in the Gn10 cells with more dramatic effects on ERK. In Gn10 cells, addition of rhGas6 under serum-free conditions stimulated ERK (13-fold) and Akt (1.5-fold) ($P < 0.05$) activity with no significant effect on ERK or Akt levels. The activation of ERK was rapid and returned to a baseline higher than control by 60 min. The activation of Akt was rapid but transient, reaching a maximum at 5 min and returning to basal by 20 min.

To confirm the increases in levels of phospho-Akt by Gas6, an alternative assessment of Akt activity was made by testing its ability to phosphorylate the substrate histone H2B in an immunocomplex kinase assay (40). Gn-10 cell lysates were harvested after exposure to serum or after serum withdrawal in the absence or presence of rhGas6. Figure 3C shows a rapid 2.3-fold increase in the phosphorylation of histone H2B by Akt at 5 min after addition of rhGas6 with a rapid return to basal levels. These data confirm that Gas6/Ark (Axl) signals through Akt in Gn10 cells.

The activity of p38 MAP kinase was also assessed in the neuronal cells, as it has recently been shown to trigger neuronal apoptosis (43) and was expected to be inhibited by Gas6 (Fig. 4). The activity of p38 MAP kinase was undetectable in Gn10 neurons after 48 h of serum deprivation in the absence or presence of rhGas6. This lack of activity was observed despite fairly high basal concentrations of the enzyme (Fig. 4). Together, these data suggest the potential importance of the ERK and PI3-K pathways and not p38 MAP kinase to transmit the Ark (Axl) signal in GnRH neuronal cells.

The Mitogen-Activated Protein/ERK Kinase (MEK) Inhibitor, PD98059, Blocks ERK Activation by Gas6

To test the hypothesis that activation of the ERK pathway is involved in Gas6/Ark rescue from neuronal programmed cell death, Gn10 cells were grown in serum or in serum-free media for 48 h. Cells were treated with vehicle, rhGas6 (400 ng/ml, 5 min), or IGF-I (100 ng/ml, 15 min). Some cells were preincubated with the MEK inhibitor, PD98059, which blocks upstream of ERK (31, 43), for 1 h before the addition of rhGas6. Cell lysates were analyzed by Western blot with ERK and phospho-ERK antibodies. Serum deprivation decreased the levels of activated ERK in the neuronal cells (Fig. 5, lanes 1 and 2), while rhGas6 triggered a 10- to 20-fold increase in levels of phospho-ERK at 5 min (lane 3). The stimulation of ERK by rhGas6 was greater than that achieved by addition of IGF-I in these neuronal cells (IGF-I, 2-fold stimulation, lane 4). The activation of ERK by rhGas6 or IGF-I was blocked in the presence of the MEK inhibitor, PD98059, to below that in unstimulated cells (lanes 6 and 7). There were no changes observed in total ERK levels after rhGas6 addition.

Inhibitors of the ERK Pathway Block Gas6/Ark Protection from Neuronal Apoptosis

To investigate the importance of the ERK-signaling pathway in Gas6/Ark protection of GnRH neuronal cells during serum withdrawal-induced cell death, Gn10 cells were grown in serum-free media in the presence or absence of rhGas6 (400 ng/ml) with or without the MEK inhibitor, PD98059 (Fig. 6). Serum withdrawal for 48 h resulted in an increase in apoptosis as measured by Hoescht staining from 2.3% to 12.3% (lanes 1 and 2). rhGas6 protected Gn10 cells from programmed cell death, decreasing levels to 4.8% (lane 3). Incubation of cells with the MEK inhibitor alone had no effect on serum withdrawal induced apoptosis (lane 4); however, Gas6 protection was lost in

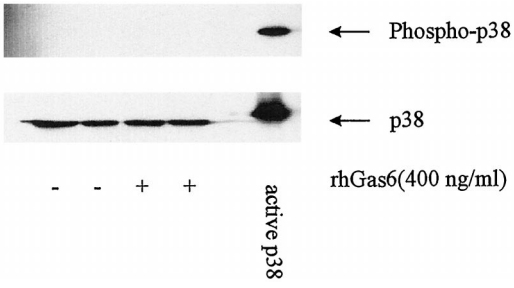

Fig. 4. Serum Deprivation and Gas6 Do Not Activate p38 MAP Kinase in Gn10 Neuronal Cells

Gn10 cells were grown in serum-free media for 48 h and then treated with vehicle or rhGas6 (400 ng/ml) for 10 min. Cell lysates were separated by electrophoresis, transferred to PVDF, and incubated with antisera specific for phospho-p38 MAP kinase and p38 MAP kinase. Lane 5 contains active p38 as a control.

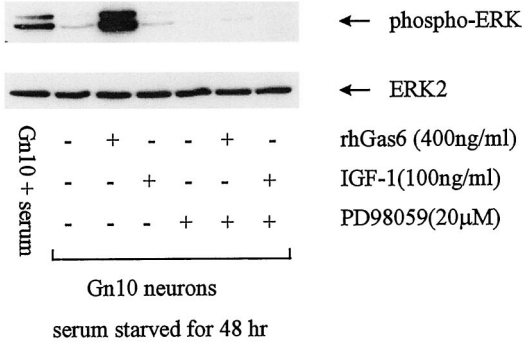

Fig. 5. The MEK Inhibitor, PD98059, Blocks ERK Activation by Gas6

Gn10 cells were serum starved for 48 h and then treated with rhGas6 (400 ng/ml) for 5 min or IGF-I (100 ng/ml) for 15 min. In some cases, the cells were pretreated with PD98059 (20 μM) for 1 h. Cell lysates were separated by electrophoresis, transferred to PVDF, and incubated with antisera specific for phospho-ERK and ERK. Lane 1, Gn10 cells grown in serum; lane 2, serum starved; lane 3, with rhGas6; lane 4, with IGF-I; lane 5, with PD98059; lane 6, with rhGas6 and PD98059; lane 7, with IGF-I and PD98059.

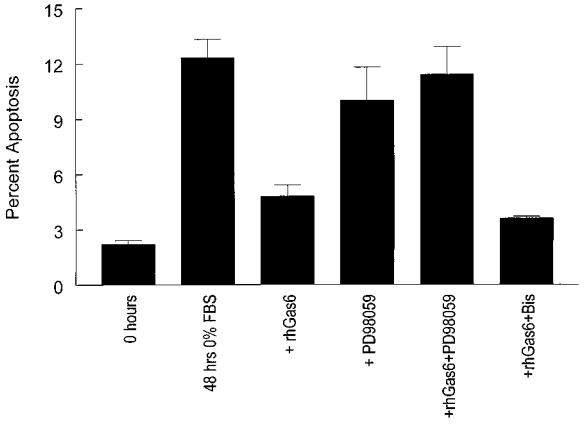

Fig. 6. Inhibition of the ERK Pathway Blocks Gas6 Rescue of Gn10 Neuronal Cells from Apoptosis

Gn10 cells were grown in serum-free media for 48 h in the absence or presence of rhGas6 (400 ng/ml) and/or the MEK inhibitor, PD98059 (20 μM), or bisindolymaleimide (100 nM). Apoptotic cells were counted by Hoescht staining. Results are the mean ± SEM of three experiments. Lane 1, Baseline apoptosis; lane 2, 48 h of serum deprivation; lane 3, with rhGas6; lane 4, with PD98059; lane 5, with rhGas6 and PD98059; lane 6, with rhGas6 and bisindolymaleimide.

the presence of the MEK inhibitor (lane 5). As another control, there was no effect of bisindolymaleimide, an inhibitor of the protein kinase C pathway (46), on Gas6 rescue of GnRH neuronal cells from growth factor withdrawal-induced cell death (lane 6). These results show the importance of the ERK pathway in Gas6/Ark (Axl) antiapoptotic signaling in GnRH neuronal cells.

Recent studies suggested that ERK activation by Gas6/Ark involves induction of mitogenesis that may indirectly contribute to protection from apoptosis (28). Therefore, Gn10 neuronal cells were grown in the presence or absence of Gas6, and cell counts were determined. There were no significant mitogenic effects of Gas6 in these neuronal cells as assessed by cell counts (data not shown). Additional studies were performed using BrdU incorporation in the absence or presence of Gas6. Cells were serum starved for 24 h and incubated in the presence or absence of rhGas6 (400 ng/ml) for 24 h. These studies also showed no effect of rhGas6 on Gn10 neuronal cells to augment BrdU incorporation (rhGas6 69.8 ± 0.13% *vs.* serum-starved control cells 100 ± 0.12%). Together, these experiments suggest that activation of the ERK pathway during Gas6/Ark rescue from neuronal apoptosis is not dependent on a mitogenic signal to trigger entry into the cell cycle and support the direct role of the pathway in protection from programmed cell death.

Wortmannin Blocks Akt Activation by Gas6

To test whether Akt is also important in Gas6/Ark protection of GnRH neurons from apoptosis, Gn10 neuronal cells were grown in serum-free media for 48 h and then treated with vehicle, rhGas6, or IGF-I as a control growth factor stimulus. Some cells were preincubated with wortmannin (1 μM) to inhibit the PI3-K pathway upstream of Akt (4). Cell lysates were analyzed by Western blot with phospho-Akt and Akt antisera (Fig. 7). Growth factor withdrawal decreased, but did not abolish, activated Akt levels (*lower band*, lanes 1 and 2). rhGas6 addition, however, consistently increased the levels of phospho-Akt by 5 min (*lower band*, lane 3). The stimulation of Akt by rhGas6 was comparable to the stimulation of Akt by IGF-I (*lower band*, lane 4). Wortmannin completely blocked the stimulation of Akt by both rhGas6 and IGF-I (lanes 6 and 7). In addition, wortmannin lowered phospho-Akt levels to below baseline, again supporting the importance of other factors in Gn10 cells that sustain an activated PI3-K via Akt signal. No changes in the protein levels of Akt were observed in the various experimental manipulations.

Inhibition of the PI3-K Pathway Blocks Gas6/Ark Rescue of Gn10 GnRH Neurons from Serum Withdrawal-Induced Apoptosis

To confirm that the changes observed in Akt activation were directly relevant to Gas6/Ark protection from neuronal apoptosis, Gn10 neurons were grown in serum-free media in the absence or presence of wortmannin (100 nM), rapamycin (20 ng/ml), rhGas6 (400 ng/ml), or combinations of the above (Fig. 8). Gn10 cells exposed to growth factor withdrawal for 48 h had an increased rate of apoptosis as determined by Hoescht staining from 2.2% to 11.7% (lanes 1 and 2). Incubation with rhGas6 partially rescued the Gn10 neurons from programmed cell death (5.4% at 48 h, lane 3). Incubation of neuronal cells with wortmannin resulted in higher levels of apoptosis (20.9%, lane 4)

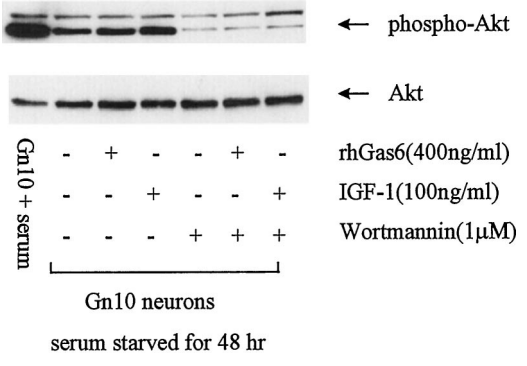

Fig. 7. Wortmannin Blocks Akt Activation by Gas6

Gn10 cells were serum starved for 48 h and treated with vehicle or rhGas6 (400 ng/ml) for 5 min or IGF-I (100 ng/ml) for 15 min in the absence or presence of pretreatment with wortmannin (1 μM) for 20 min. Cell lysates were separated by electrophoresis, transferred to PVDF, and incubated with phospho-Akt and Akt antisera. Lane 1, Gn10 cells grown in serum; lane 2, serum starved; lane 3, with rhGas6; lane 4, with IGF-I; lane 5, with wortmannin; lane 6, with rhGas6 and wortmannin; lane 7, with IGF-I and wortmannin.

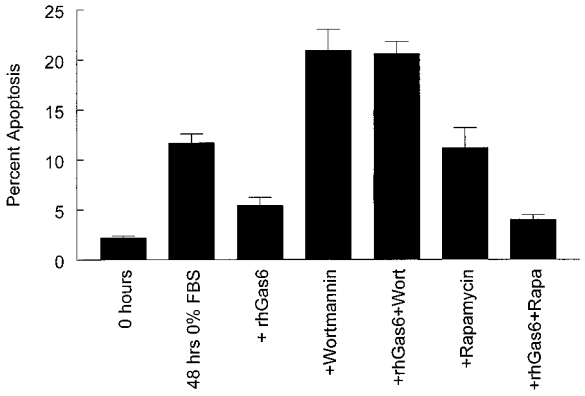

Fig. 8. Inhibition of the PI3-K Pathway Blocks Gas6 Rescue of Gn10 Neurons from Serum Withdrawal-Induced Apoptosis

Gn10 neurons were grown in serum-free media for 48 h in the absence or presence of rhGas6 (400 ng/ml), wortmannin (100 nM), rapamycin (20 ng/ml), or combinations of the above. Cells were harvested and rates of apoptosis determined by staining with Hoescht 33258. Results are the mean ± SEM of three experiments. Lane 1, Baseline apoptotic cells in Gn10 cells; lane 2, 48 h in serum-free media; lane 3, with rhGas6; lane 4, with wortmannin; lane 5, with rhGas6 and wortmannin; lane 6, with rapamycin; lane 7, with rhGas6 and rapamycin.

compared with serum withdrawal (11.7%). These results suggest the PI3-K pathway is used by other membrane receptors to protect GnRH neurons from cell death in addition to those controlling apoptosis due to growth factor deprivation. rhGas6 was unable to protect the neuronal cells in the presence of the wortmannin blockade of the PI3-K pathway (lane 5). There was no effect of rapamycin, a selective inhibitor of S6 kinase that is also downstream of PI3-K (47), on the rates of apoptosis (lane 6). In addition, rapamycin did not influence the ability of rhGas6 to rescue from neuronal apoptosis (lane 7), suggesting that Akt and not S6 kinase is the important downstream target of PI3-K in these cells. Together, these data support the hypothesis that Gas6/Ark protection of GnRH neurons is mediated through mechanisms that involve Akt as well as ERK.

DISCUSSION

Programmed Cell Death in GnRH Neurons

During development, apoptosis plays a critical role in determining the ultimate fate of neuronal populations. Studies have estimated that 20–80% of the neurons expressed during embryogenesis die before maturation of the organism (34). The GnRH neuronal population is unique in that, unlike other neurons that express hypothalamic releasing factors, the neurons must migrate from the olfactory placode into the forebrain. In addition, the population is small, with only 800–1000 neurons in the rodent making the journey (2–5). Because of the ultimate importance of appropriately targeting this subpopulation of neurons into the hypothalamus for reproductive competence, one might expect a complex series of mechanisms to modulate the rate of programmed cell death during their migratory journey. The ability to identify factors involved in this process has been limited by the small size of the neuronal population and difficulty in working with primary cultures. We took advantage of two available GnRH-producing neuronal cell lines to use differential display-PCR to clone candidate cDNAs that might play a role in GnRH neuronal migration or gene expression (14). Ark, expressed in Gn10 GnRH cells derived when GnRH neurons are migrating, is the first candidate to be studied functionally. The potential role of Ark (Axl) in cell-cell contact has been suggested in 32D myeloid cells (23–27) and in vascular smooth muscle cells (27), but it has not been studied in neuronal cells. The role of Ark (Axl) in the modulation of GnRH gene expression is under active investigation in the laboratory (48). The present study strongly supports Ark's role in protection of the GnRH neuronal population from programmed cell death.

Since Ark is not expressed in the GT1–7 cells derived from GnRH neurons in the forebrain, one might expect that additional protective mechanisms against apoptosis of the GnRH population are not needed once the appropriate targeting has taken place. Gas6 is not expressed in these neuronal cells, so one would hypothesize that adjacent glia or neuronal cells synthesize the Ark (Axl) ligand during the migratory process. This is consistent with an increasing literature supporting the role of glial elements in neuronal migration and survival (49).

The Functional Role of Ark to Protect from Programmed Cell Death

Serum deprivation is a classic model system to study programmed cell death. Although most studies of the Ark (Axl) family have been performed in this model system, Bellosta *et al.* (21) recently reported that Gas6/Ark signaling protects fibroblasts from apoptosis induced by tumor necrosis factor-α and c-Myc but not that induced by UV irradiation or staurosporine. Similar studies of Ark function in neuronal cells using these alternative model systems of apoptosis have not yet been performed. To support the physiological importance of Ark (Axl), however, embryonic fibroblasts from Ark (Axl) knockout mice were found to be more susceptible to growth factor withdrawal-induced apoptosis than wild-type cells (21). Together, these data suggest the general role of Ark (Axl) in modulating the rate of programmed cell death.

Gas6/Ark Signaling in Neuronal Cells Is Different Than in Other Systems

Ark was initially derived in studies searching for novel growth factor receptors, and Axl has been found to be

overexpressed in some leukemia cell lines (18, 27). Initial studies focused on the role of the kinase as a mitogenic stimulus, but recent studies have suggested Ark (Axl) is only a weak mitogen unless overexpressed (22, 28). In GnRH neuronal cells, Gas6/Ark (Axl) signaling is not associated with a mitogenic response, which is expected since neuronal cells are not subject to significant proliferative responses.

In the GnRH neuronal cell lines, both the ERK and Akt pathways were activated in the presence of serum. The Gn-10 (Ark positive) cells, however, had higher levels of activated ERK and Akt than the GT1–7 (Ark negative) cells, suggesting that the high endogenous levels were not due solely to SV_{40} TAg immortalization. In addition, growth factor withdrawal decreased activated ERK levels dramatically with less effect on activated Akt levels, supporting the recent observations that many neuronal cells use the PI3-K via Akt pathway as the major control point for multiple endogenous signals that mediate neuronal survival (38, 40, 45).

The ERK intracellular signaling system has been associated with both proapoptotic and antiapoptotic effects (reviewed in Ref. 32). Gardner and Johnson (33) demonstrated that fibroblast growth factor 2 suppression of tumor necrosis factor-α mediated apoptosis required Ras activation of ERK in L929 cells. Párrizas *et al.* (44) showed the activation of both ERK and PI3-K pathways in IGF-I-mediated rescue from apoptosis in differentiated PC12 cells (44). Bellosta and co-workers (21) have shown in NIH-3T3 cells that Ark triggered a modest increase in ERK activity that accompanied the increased survival of cells at concentrations that did not promote DNA synthesis. In our studies, incubation with the MEK inhibitor (PD98059) completely reversed Gas6 protection from programmed cell death in GnRH neuronal cells. These data show the critical role of the ERK pathway in transmitting the Gas6 signal from the membrane to the appropriate intracellular targets to rescue these neuronal cells from growth factor withdrawal-induced apoptosis. In contrast to Gas6/Ark, IGF-I triggered activation of Akt to a greater extent than ERK. These results confirm the cell specificity of intracellular signaling pathways downstream of growth factor receptors.

In prior studies of other downstream signaling pathways activated by Gas6/Ark (Axl), divergent results have been reported (22, 28, 29). Goruppi and coworkers (29) have suggested the importance of PI3-K working through S6K as well as Src activation in both mitogenic and survival activities by Gas6/Axl signaling in NIH-3T3 cells. The data concerning Src activation are complex since the Axl cytoplasmic domain lacks Src consensus binding sequences, and Src could not be coimmunoprecipitated with the receptor (29). Since the NIH-3T3 cells contain Tyro3, another family member that heterodimerizes with Ark (Axl), the complement of protein partners in each cell may influence the importance of different signaling pathways. However, the GnRH neuronal cells do not express Tyro3 (X. Xiong and M. E. Wierman, unpublished observations), suggesting the observed responses are due solely to Ark (Axl) activation.

Goruppi and colleagues (29) also found that rapamycin treatment inhibited the ability of Gas6 to prevent apoptosis-associated Gas2 proteolytic cleavage. In our studies, rapamycin had no effect on Gas6 protection from apoptosis, in contrast to wortmannin. Together with the data showing increased phospho-Akt and phosphorylation of the Akt substrate, histone H2B, by Gas6, these results suggest that in GnRH neurons, the PI3-K pathway triggers Akt as an additional downstream effector to modulate the sensitivity of GnRH neurons to trophic factor withdrawal. The recent demonstration that Akt inhibits apoptosis in cerebellar neurons (40), in rat hippocamal H19–7 neuronal cells (45), and in sympathetic neurons (38) and that Akt also phosphorylates the proapoptotic BCL-2 member, BAD, in neuronal cells to inactivate it (42) are all supportive of the major importance of this pathway in mediating multiple receptor-mediated antiapoptotic signals. Since blockade of either the PI3-K or the ERK pathways reversed the protective effect of Gas6, it is unclear whether these are parallel independent signaling systems or whether there is cross-talk at some level between the components. Future studies will be needed to investigate these possibilities.

MATERIALS AND METHODS

Cell Culture

GT1–7 and Gn10 GnRH neuronal cells were grown in DMEM supplemented with 5% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). For serum starvation, cells were plated at 60% confluency, grown overnight, and changed to serum-free media, and the incubation was continued for 6–72 h depending on the experimental design.

Reagents

Wortmannin [a specific inhibitor of PI3-K (50)], rapamycin [a specific inhibitor of p70 S6 kinase (47)], bisindolymaleimide [a specific inhibitor of protein kinase C (46)], and insulin-like growth factor 1 (IGF-I) were purchased from Sigma Chemical Co. (St. Louis, MO). PD98059 (an inhibitor of MEK1 and MEK2, the upstream regulators of ERK) was purchased from New England Biolabs, Inc. (Beverly, MA) Purified IgG Ark#318 was raised against the Ark extracellular domain (21). Antibodies specific to Akt and phospho-Akt were purchased from New England Biolabs, Inc. Antiactive MAP kinase pAb (phospho-ERK) and anti-active p38 antibodies were purchased from Promega, Inc. (Madison, WI). Antisera specific to ERK2 and p38 MAP kinase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human Gas6 (rhGas6) from Amgen (Thousand Oaks, CA) was used in all studies (26).

Quantification of Apoptotic Cells

Neuronal cells were grown in standard conditions with or without FCS for various time intervals. For Hoescht staining, cells were fixed on dishes or polylysine-coated slides in 1% paraformaldehyde for 2 min at room temperature followed by

70% ethanol in glycine buffer (100 mM, pH 3.0) for 20 min at −20 C. After fixation, the cells were washed with PBS three times and then incubated in Hoescht 33258 stain (8 μg/ml in PBS) for 15 min at room temperature. The cells were washed with PBS three times and then stored in the dark immersed in PBS. The stained cells were viewed under a fluorescent microscope (Olympus IMT-2 inverted microscope, Olympus Corp., Lake Success, NY). Apoptotic cells were measured by counting the number of neuronal cells with condensed or fragmented chromatin. These cells typically appeared small and rounded and bright green. One thousand cells were counted from eight randomly chosen fields. The rate of apoptosis was expressed as a percentage of total counted cells.

For ethidium bromide/acridine orange staining, cells (floating and attached) were resuspended in cold PBS. A 1:2 dilution of 100 μg/ml solutions of ethidium bromide and acridine orange was mixed with the resuspended cells. The stained cells were viewed as above. Apoptotic cells were scored as those with orange-red fragmented, condensed nuclei. The rate of apoptosis was expressed as a percentage of total counted cells.

Western Blot Analysis

Three million GT1–7 or Gn10 neuronal cells were grown for 48 h in standard media with or without serum. After the experimental manipulation, cells were washed twice with PBS (4 C), and lysed in 250–500 μl of cell lysis buffer containing 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 1% glycerol, 1 mM dithiothreitol, one protease inhibitor tablet/50 ml (Boehringer Mannheim, Indianapolis, IN), and freshly added 20 mM Na_3VO_4, 25 mM NaF, and 20 mM sodium pyrophosphate at 4 C. Lysed cells were sonicated (Branson sonifier 250, at power 3, Branson Sonic Power Co., Danbury, CT) for 10 pulses. The lysate was then spun at 14,000 × *g* for 10 min at 4 C. The supernatant was then assayed for protein concentration with the BCA protein assay kit (Pierce, Inc., Rockford, IL). An aliquot of 20–30 μg of total protein was resolved by SDS/PAGE on 10–12% gels using a Bio-Rad mini-gel system (Bio-Rad Laboratories, Richmond, CA). Subsequently, proteins were transferred to Hybond polyvinylidene difluoride (PVDF) (Amersham Lifesciences, Inc., Arlington Heights, IL) at 100 V for 1 h at 4 C. The membranes were blocked in 5% milk, TBS-T buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) overnight at room temperature. Protein present on the blot was visualized using enhanced chemiluminescence (ECL) immunodetection reagents (Amersham Life Sciences, Inc.). For each antiserum, the primary antibody was diluted to 1:500–1:2000 and incubated with the membrane at room temperature for 2 h. The secondary antibody was diluted 1:2000–1:3000 and incubated with the membrane for 1 h at room temperature. An additional four washes were performed before immunodetection according to the manufacturer's instruction (Amersham Lifesciences, Inc.).

Immune-Complex Kinase Assay for Akt Activity

Gn10 cells were growth for 48 h in normal growth media or serum-free media. The serum-starved cells were incubated with 400 ng/ml rhGas6 for various times, washed once with PBS (4 C), and lysed in 137 mM NaCl, 10% glycerol, 1% NP-40, 20 mM Tris, pH 8, one protease inhibitor tablet/50 ml, 20 mM Na_3VO_4, 25 mM NaF, and 20 mM sodium pyrophosphate at 4 C. The lysed cells were sonicated and spun as described above. Total protein (2–5 mg) was immunoprecipitated with 10 μg of antihuman Akt-1 pleckstrin homology domain antibody (Upstate Biotechnology, Lake Placid, NY) for 1.5 h at 4 C with constant mixing. Subsequently, 100 μl of protein A/G agarose (Calbiochem, La Jolla, CA) was added and the incubation continued for 1 h. The immune complexes were washed three times in lysis buffer, once in dH_2O, and once in kinase assay buffer minus ATP (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$) at 4 C. Akt kinase activity was determined by incubating the immune complexes with 2 μg histone H2B, 10 μCi [γ^{32}P]ATP, 5 μM ATP, and kinase assay buffer for 30 min at room temperature. The supernatants were resolved by 15% SDS-PAGE, and the phosphorylation of histone H2B was detected by autoradiography.

Northern Analyses

Twenty micrograms of total RNA isolated from GT1–7 and Gn10 neuronal cells were separated by electrophoresis in a 1.4% formaldehyde agarose gel transferred to nitrocellulose and baked at −80 C as previously described (14). The blot was hybridized with ^{32}P-radiolabeled Ark cDNA (15), washed, and exposed to film at −70 C for 1–2 days.

BrdU Labeling for Cell Proliferation

Gn10 neuronal cells were grown in serum-free media for 24 h and then incubated with vehicle or rhGas6 (400 ng/ml) for 24 h. Cells were then labeled with BrdU for 2–4 h using the cell proliferation enzyme-linked immunosorbent assay (ELISA) colorometric kit from Boehringer Mannheim. BrdU incorporation was assessed by colorometric assay using a Biotek ELISA reader. Data represent the mean ± SEM of six to eight dishes.

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1999 Gordon Research Conference on Hormone Action: Preliminary Program

July 25–July 30, 1999, Kimball Union Academy, Meriden, New Hampshire

Holly A. Ingraham, UCSF, Chair

Gary L. Firestone, UCB, Vice-Chair

Sunday, July 25

7:30 pm **Opening lecture:** ***To be Announced***

Monday, July 26

9:00 am **Cell Signaling in Development**
Discussion leader: **Sally Camper**, Univ. Michigan, Ann Arbor
Gail Martin, UCSF, CA, *FGF Signaling*; **Mike German**, UCSF, CA, *Molecular Determinants of Pancreatic Development*; **Gary Ruvkun**, Harvard Medical School, Boston, MA, *Metabolic Signaling in Invertebrates*; **Phil Beachy**, Johns Hopkins, Baltimore, MD, *Hedgehog Signaling in Vertebrate Development.*

4:00 pm **Poster Session Group I**

7:30 pm **Signaling and Cross Talk**
Discussion leader: **Gary Firestone**, UCB, CA
Stephen Foord, Glaxo Wellcome Medicines Research Center, Hertfordshire, UK, *RAMPs and G-Protein Receptor Specificity*; **Michael Karin**, UCSD, CA, *Protein Kinase Cascades and the Pro-inflammatory Response*; **Tom Wilke**, UT Southwestern, TX, *RGS Proteins and Ca^{2+} in Signaling Specificity.*

Tuesday, July 27

9:00 am **Technical Advances in the Discovery of Peptides and Receptors**
Discussion leader: **Wylie Vale**, The Salk Institute, La Jolla, CA
Baldomero Olivera, University of Utah, Salt Lake City, UT, *Conus Peptides: Biomedical Applications and Insights into Neuropharmacological Principles*; **Roger Cone**, Vollum Institute, Portland, OR, *Peptide Signaling in Metabolism*; **Masashi Yanagisawa**, UT, Southwestern Medical School/HHMI, Dallas, TX, *Discovery of New Peptides Regulating Food Intake and Metabolism*; **David Julius**, UCSF, CA, *Discovery of New Receptors in the Sensory System.*

4:00 pm **Poster Session Group II**

7:30 pm **Hormones and Behavior**
Discussion leader: **Kelly Mayo**, Northwestern University, Evanston, IL
Catherine Woolley, Northwestern University, Evanston, IL, *Estrogen and the Hippocampus*; **Allison Doupe**, UCSF, CA, *Hormones and Imprinting in Birdsong Acquisition*; **Richard Axel**, Columbia University/HHMI, New York, NY, *Pheromones in Wiring of the Reproductive Circuitry*

Wednesday, July 28

9:00 am **Structure and Transcription by Nuclear Receptors**
Discussion leader: **Chris Glass**, UCSD, CA
Tom Scanlan, UCSF, CA, *Chemistry and Biology of Thyroid Receptor Action*; **Michael G. Rosenfeld**, UCSD/HHMI, CA, *Integration of Extracellular Signaling and Nuclear Receptor Function*; **Keith Yamamoto**, UCSF, CA, *Signals and Surfaces Affecting Intracellular Receptor Function*; **Robert Roeder**, Rockefeller University, New York, NY, *Transcriptional Regulation by Nuclear Receptors.*

7:30 pm **In Vivo Models for Probing Steroid Actions**
Discussion Leader: **Bill Schrader**, Ligand Pharmaceuticals, San Diego, CA
David Russell, University of Texas, SW Medical Center, Dallas, TX, *In Vivo Mutants of Steroid Synthesis in Reproduction*; **Gunther Schütz**, German Cancer Research Center, Heidelberg, Germany, *Designer Receptors for Probing Function of Glucocorticoids*; **Jeffrey Miner**, Ligand Pharmaceuticals, San Diego, CA, *Novel Non-Steroidal Therapeutic GR Ligands.*

Thursday, July 29

9:00 am **Orphan Nuclear Receptor Function**
Discussion Leader: **Keith Parker**, UTSW Medical Center, Dallas, TX
Yoel Sadovsky, Washington University, St. Louis, MO, *SF-1 in Reproductive and Adrenal Physiology*; **David Moore**, Baylor College of Medicine, Houston, TX, *SHP, A Novel Orphan Nuclear Receptor*; **Larry Jameson**, Massachusetts General Hospital, Boston, MA, *Dax-1 in Adrenal Function and Sex Determination*; **Frances Sladek**, UCR, CA, *HNF-4, Insights into Liver Function and Diabetes.*

7:30 pm **Nuclear Receptor Function**

Ron Evans, Salk Institute/HHMI, San Diego, CA, *Nuclear Receptor Function in Adipose Development*; **Bert O'Malley**, Baylor College of Medicine, Houston, TX, *Nuclear Receptor Cofactors and Transcription.*