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The purpose of this research project was originally to characterize growth inhibitory effects of interleukin-4 (IL-4) in breast cancer cells and identify key signaling molecules that may be targets for future strategies to enhance the negative growth effects of IL-4. The original investigators have reported that IL-4-mediated growth inhibition is associated with increased apoptosis and that IL-4 activates two important signaling molecules, IRS-1 and STAT6. Inhibition of IRS-1 does not block IL-4-mediated growth effects while inhibition of STAT6 decreases IL-4-mediated growth inhibition and apoptosis. Additionally, over-expression of STAT6 mimics the effect of IL-4. Finally, the investigators examined the mechanism of INF gamma-mediated growth inhibition of breast cancer cells in vitro. In addition to the JAK-STAT pathway, INF gamma decreases breast cancer cell growth in p21 dependent and independent pathways. In conclusion, their research on the mechanism and signal transduction pathways of IL-4 in human breast cancer cells has resulted in numerous original and significant findings.

As of 10/00, this grant entitled "Overexpression of IL-4 Signaling Pathway to Inhibit Breast Tumor Growth" was transferred to Jill Ricono, a graduate student in the Department of Nephrology at the University of Texas Health Science Center at San Antonio where research focuses on the pathogenic mechanisms involved in kidney development and progression of renal disease; specifically, the effects of Platelet-derived growth factor (PDGF) on development and maturation of microvascular cells and their precursors in the metanephric kidney.

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Introduction

The purpose of this research project was originally to characterize growth inhibitory effects of interleukin-4 (IL-4) in breast cancer cells and identify key signaling molecules that may be targets for future strategies to enhance the negative growth effects of IL-4. The original investigators have reported that IL-4-mediated growth inhibition is associated with increased apoptosis and that IL-4 activates two important signaling molecules, IRS-1 and STAT6. Inhibition of IRS-1 does not block IL-4-mediated growth effects while inhibition of STAT6 decreases IL-4-mediated growth inhibition and apoptosis. Additionally, over-expression of STAT6 mimics the effect of IL-4. Finally, the investigators examined the mechanism of INF gamma-mediated growth inhibition of breast cancer cells in vitro. In addition to the JAK-STAT pathway, INF gamma decreases breast cancer cell growth in p21 dependent and independent pathways. In conclusion, their research on the mechanism and signal transduction pathways of IL-4 in human breast cancer cells has resulted in numerous original and significant findings.

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Final summary

The project title of this research grant is "Overexpression of IL-4 signaling pathway to inhibit breast tumor growth" that had been funded for the performance period of July 1998- August 2001. The original principle investigator, Jennifer Gooch, has successfully defended her dissertation entitled "The role of IRS-1 in IGF-1 and IL-4-mediated growth effects in human breast cancer cells" in September of 1999 while being supported by this DOD funded research project. As of October 2000, this same grant had been approved to be transferred to Jill Ricono, a graduate student in the Department of Nephrology at The University of Texas Health Science Center at San Antonio where Dr. Gooch is now employed.

In the annual summary reports for 1998-2000, Dr. Gooch reported significant progress on this project including publication of several key findings and the completion of specific aim 1. These investigators were the first to describe that apoptosis was the mechanism involved in IL-4 induced growth inhibition in human breast cancer cells [1]. They next examined the signaling pathways activated by IL-4 in human breast cancer cells in vitro and determined which signaling molecules are required for IL-4-mediated growth effects. They found that IL-4 activates two important signaling molecules, IRS-1 and STAT6. Using an inducible anti-sense strategy to inhibit IRS-1 expression in MCF-7 breast cancer cells, they showed that IRS-1is not required for IL-4-mediated growth inhibition and apoptosis. However, cells expressing anti-sense IRS-1were less sensitive to IGF-I (insulin like growth factor-I)-induced proliferation and protection from apoptosis [2]. Furthermore, they demonstrated that IL-4 failed to inhibit growth of MCF-7 cells

when a dominant-negative STAT6 is expressed in the cells, whereas over-expression of a full-length STAT6 resulted in growth inhibition [3].

The observation that IL-4 induced apoptosis in breast cancer cells was made only after multiple tests for apoptosis were confirmed, e.g., appearance of pyknotic nuclei, chromosome condensation, cleavage of poly-ADP-ribose Polymerase, appearance of sub-G1 DNA content using FACS sorting, and degradation of DNA into internucleosomal fragments (DNA laddering). However, published reports of DNA laddering in some cells, including MCF-7 breast cancer cells, have been controversial. The investigators here show that the ability of MCF-7 cells to undergo apoptosis is strain specific. They found that when 6 different strains of MCF-7 cells that were obtained by different laboratories were treated with adriamycin, only three of the strains produced DNA laddering [4], suggesting that DNA laddering may not only be cell type specific, but may also differ between strains of cells of the same cell type.

IL-4 and IGF-I share common signaling molecules, namely IRS-I. In contrast to IL-4, IGF-I induces proliferation and increases survival of breast cancer cells. Although the investigators' work eventually demonstrated that IRS-1 is not required for IL-4-induced growth effects, growth effects mediated by IGF-I via IRS-1 were explored. The investigators showed that IGF-I increased survival and decreased apoptosis of MCF-7 breast cancer cells after treatment with either doxorubincin or paclitaxel, both chemotherapy drugs [5]. Interestingly, they found differential effects of the anti-cancer drugs mediated by IGF-I. They reported that IGF-I's protective effect following dexorubicin-induced cell death required activation of PI-3 kinase and Erk1/2 MAP kinase while IGF-I's protective effect following paclitaxel required activation of only Erk1/2 MAP kinase. As PI-3 kinase has been implicated in inhibition of apoptosis and Erk1/2 is

commonly associated with proliferation, it is apparent that IGF-I can both decrease apoptosis and increase proliferation to enhance cell survival.

The investigators also characterized the effects of interferon (INF) gamma on breast cancer cell lines. They found that INF gamma inhibits growth of several cell lines and that this inhibition is associated with changes in the cell cycle, mainly an increase of in G1. However, INF gamma did not inhibit anchorage independent growth of one cell line, MDA-MB-231, which was associated with a lack of p21, the cyclin dependent kinase inhibitor. Therefore, INF gamma inhibits the growth of breast cancer cells and utilizes both p21-dependent and p21-independent pathways[6].

As previously mentioned, the grant entitled "Overexpression of IL-4 signaling pathway to inhibit breast tumor growth" had been approved to be transferred to Jill Ricono, a graduate student in the Department of Nephrology at The University of Texas Health Science Center in San Antonio. Research in this laboratory focuses on the pathogenic mechanisms involved in kidney development and progression of renal disease. The role of growth factors and chemokines in mediating these processes with major emphasis on platelet-derived growth factor (PDGF) is being explored. Studies in cultured embryonic and adult kidney cell lines aim at identifying membrane, cytoplasmic and nuclear signals involved in mediating biologic effects of PDGF and specifically the role of PDGF in mesangial cell development. My research in this laboratory has focused on the effects of PDGF on development and maturation of microvascular cells, including mesangial cells, and their precursors in the metanephric kidney.

Since biologically active PDGF is a dimer, binding of PDGF ligand involves dimerization of the receptor, forming either heterodimers, $\alpha\beta$, or homodimers, $\alpha\alpha$ or $\beta\beta$.

The different PDGF receptors have different binding capacities. PDGFRα binds PDGF-AA, -AB, -BB and -CC ligand isoforms, with binding of PDGF-AA with higher affinity than -BB, whereas PDGFRβ binds only PDGF-BB and –DD with high affinity (Heldin et al., 1989; Heldin and Westermark, 1989; Seifert et al., 1993; Li et al., 2000; Bergsten et al., 2001). Studies utilizing mice carrying mutations in either PDGF B-chain ligand (Leveen et al., 1994) or PDGFR β (Soriano, 1994) conclusively demonstrate the role of these factors in the development of mesangial cells within the glomeruli. In both studies PDGFR α was intact and functional, yet it was not able to compensate for loss of the PDGFRβ. Therefore, we hypothesized there is differential signaling events transduced by PDGF AA and BB through their cognate receptors that lead to proliferation and migration in metanephric mesenchymal (MM) cells. To test this hypothesis, metanephric blastemas were isolated from wild type and PDGFRb-deficient (-/-) embryos at 11.5 days post conception, the time when metanephrogenesis is initiated. Cell lines were established from the isolated metanephric blastemas and characterized [7]. First we investigated the biologic effects and signaling pathways of PDGF AA and PDGF BB in MM cells. Addition of PDGF BB to cultured wt MMC increases DNA synthesis as measured by 3H-thymidine incorporation. PDGF AA was not mitogenic indicating that activation of alpha receptor is not sufficient for this biological response. PDGFRβ-deficient MMC did not respond mitogenically to either isoform, PDGF AA or BB [8]. Addition of PDGF BB to wt MMC also increased migration of these cells, whereas PDGF AA had no effect on cell migration. However, PDGF AA did enhance migration of -/- MMC [9]. To investigate the mechanism of this differential effect, we studied the signaling molecules activated by PDGF AA or BB in wt or -/- MM cells. In both cell

lines, wt and -/- MMC, both PDGF AA and PDGF BB activated ERK1/2 MAP kinase, PI3-kinase, and Akt/PKB signaling pathways [10]. However, we found two pathways that are differentially influenced by PDGF AA or BB. PDGF BB, but not AA, increases the concentration of Ca^{2+} in wt MMCs, whereas AA or BB did not have any effect in -/- MMCs. Similarly, BB, but not AA, stimulates diphenylene iodonium (DPI)-inhibitable superoxide anion production, and NADPH oxidase activity as well as an increase in intracellular hydrogen peroxide (H_2O_2) levels in wt cells [11]. Therefore, we postulated that Ca^{2+} and reactive oxygen species (ROS) may be part of a signaling pathway leading to suppression of AA-induced migration in wt MMCs through PDGFRb. To assess the involvement of Ca^{2+} in migration we used chelators of extracellular Ca^{2+}, and to evaluate the role of ROS, we used the antioxidant N-acetyl cysteine (NAC). In summary, chelation of extracellular Ca^{2+} or ROS, the two signals specifically related to the PDGFR β, restores AA-induced migration in wt MMCs, similar to its effects in AA-induced migration in -/- MMCs. The stimulation of ROS or Ca^{2+} release in the -/- MMCs nullifies AA-induced migration, as seen in wt MMCs. Taken together, these results suggest that ROS and Ca^{2+} play an essential role in suppressing PDGF AA-induced migration in wt MMCs [12].

To further confirm the hypothesis that signals from the PDGFRβ may suppress PDGF AA-induced migration, we transfected wild type PDGFRβ into the -/- MM cells and made stable cell lines. The addition of PDGFRβ restored PDGF BB-induced DNA synthesis and migration, while PDGF AA-induced migration was suppressed [13]. When Vector alone was trasfected into the -/- MM cells, PDGF AA induced migration, as in -/- MM cells alone. We have also transfected PDGFR mutant (F5) lacking five critical cytoplasmic tyrosine residues implicated in RasGAP, PI3-K, SHP2, and PLC-g signaling and a panel of "add-back" mutants that restore one

of the tyrosine residues that can bind only one of the receptor-associated proteins. Stable cell lines were made and studies to determine if the PDGFRβ mutants can restore the wild type phenotype are in progress. Also, as mentioned previously, we have shown Ca^{2+} and ROS to be signaling molecules that are specific to PDGFRβ in MM cells and have identified NADPH oxidase-derived ROS to act as upstream mediators of Ca^{2+} in the signaling pathway linking PDGFRβ to the repression of AA-induced migration. In addition, we are in the process of identifying which homolog of the neutrophilic catalytic subunit of the NA(D)PH enzyme (gp91phox) may be present in MM cells. In -/- MM cells, the basal levels of ROS production are significantly higher than the basal levels of ROS production in wt MM cells. We have demonstrated that the mRNA levels of Nox4 (for NAD(P)H oxidase 4, a homolog of gp91phox) is much higher in -/- cells than in wt MM cells [14]. However, transfecting the antisense oligonucleotide of Nox4 into MM cells did not reduce ROS levels suggesting ROS prodution may be dependent upon another homolog of gp91phox. Also, PDGF may be down regulating the Nox4 message in the wt MM cells, although these data are not conclusive at this time. Therefore, we are in the process of identifying other Nox homologs that may be responsible for the PDGF BB-induced production of ROS.

In conclusion, our work on this DOD funded research project has been exciting and fruitful. We have contributed to knowledge about the development of mesangial cells and the involvement of PDGF during nephrogenesis. Also, the previous investigators have published original papers in reputable journals and have reported many exciting findings regarding the role of IL-4 and related signaling pathways in breast cancer cells. I would like to thank you for your support of this project during my doctoral training.

Key Research Accomplishments

1. Interleukin-4 inhibits growth of breast cancer cell lines and induces apoptosis.
2. IL-4 activates IRS-1 and JAK-STAT signaling pathways in breast cancer cell lines.
3. Inhibition of STAT6 blocks IL-4 mediated growth inhibition and apoptosis and overexpression STAT6 increases IL-4-mediated growth effects.
4. The appearance of DNA ladders does not correspond to the induction of apoptosis in different strains of MCF-7 cells.
5. IGF-I is a potent survival factor for MCR-7 breast cancer cells and acts by both increasing proliferation and decreasing apoptosis.
6. Interferon gamma also inhibits the growth of breast cancer cells and utilizes both p21-dependent and independent pathways.
7. Cell lines were established from isolated wild type and PDGFRβ-deficient metanephric blastemas and characterized.
8. PDGF BB is mitogenic in wild type MM cells whereas PDGF AA is not mitogenic in neither wild type nor PDGFRβ-deficient MM cells, suggesting PDGFRβ is necessary for PDGF-induced proliferation.
9. PDGF BB induced migration in only wild type MM cells, whereas PDGF AA induced migration only in PDGFRβ-deficient MM cells.
10. In both cell lines, wild type and PDGFRβ-deficient MM cells, PDGF AA and -BB activate Erk1/2 MAP kinase, PI3-kinase and Akt/PKB signaling pathways.
11. PDGF BB, but not PDGF AA, increases the concentration of Ca^{2+} and stimulates reactive oxygen species in wild type MM cells.

12. By using chelators of Ca^{2+} or scavengers of ROS, our results suggest that Ca^{2+} and ROS play an essential role in suppressing PDGF AA-induced migration in wild type MM cells.

13. The addition of wild type PDGFRβ restored PDGF BB-induced DNA synthesis and migration, while PDGF AA-induced migration was suppressed.

14. mRNA levels of Nox4 is much higher in PDGFRβ-deficient MM cells than in wild type MM cells. Also, basal levels of ROS production in PDGFRβ-deficient MM cells are significantly higher than basal levels of ROS production in wild type MM cells.

Reportable Outcomes

Manuscripts:

1. G Ghosh Choudhury, JM Ricono. Synergistic Action of interfong (IFNg) on PDGF-induced DNA Synthesis is mediated via c-fos gene transcription in mesangial cells (MC). *Bioch. Biophys. Res. Commun.* Jul 14;273(3):1069-77, 2000.
2. JM Ricono, M Arar, GG Gosh Choudhury, HE Abboud. Differential Mitogenic Signaling of Platelet-derived Growth Factor (PDGF) Isoforms In Rat Metanephric Mesenchymal Cells. *American Journal of Physiology-Renal.* 282:F211-F219, 2002.
3. JL Gooch, YTang, JM Ricono, HE Abboud. Insulin-like growth factor-I induces renal hypertrophy via a calcineurin-dependent mechanism. *Journal of Biological Chemistry.* Vol.276, pp. 42492-42500. 2001.
4. JM Ricono, Y-C Xu, M Arar, D-C Jin, JL Barnes, HE Abboud. Morphological insights into the origin of glomerular endothelial and mesangial cells. Submitted. 2001.
5. Y Gorin, JM Ricono, N-H Kim, B Bhandari, G Ghosh Choudhury, HE Abboud. Role of Rac1 in Akt/Protein Kinase B activation by angiotensin II. Submitted. 2001.
6. JM Ricono, Y Gorin, G Ghosh Choudhury, P Soriano, A Kazlauskas, HE Abboud. PDGF receptor β inhibits PDGF AA-induced migration in metanephric mesenchymal cells. In preparation.
7. Y Gorin, JM Ricono, N-H Kim, B Bhandari, G Ghosh Choudhury , HE Abboud. Differential effects of angiotensin II on ERK1/2 and p38-MAPK in mesangial cells: Involvement of arachidonic acid, Rac1 and reactive oxygen species. In preparation.
8. Gooch JL, Herrera R, Yee D. The role of p21 in IFN-gamma-mediated growth inhibition in human breast cancer cells. *Cell Growth and Differentiation*, 6:335-342, 2000.
9. Lee AV, Gooch JL, Osterreich S, Guler B, Yee D. IGF-I-induced degradation of IRS-1 is mediated by the 26S proteosome and requires PI-3 kinase. *Molecular Cell Biology*, 5:1489-1496, 2000.
10. Gooch JL, Yee D. Strain-specific differences in the formation of apoptotic DNA ladders in MCF-7 breast cancer cells. *Cancer Letters*, 144:31-37, 1999.
11. Gooch JL, Van Den Berg CL, Yee D. Insulin-like growth factor (IGF) -I rescues breast cancer cells from chemotherapy-induced cell death: proliferative and anti-apoptotic effects. *Breast Cancer Research and Treatment,* 56:1-10, 1999.
12. Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, Yee D. Enhancement of the insulin-like growth factor signaling in human breast cancer: Estrogen regulation of insulin receptor substrate-1 (IRS-1) in vitro and in vivo. *Molecular Endocrinology*, 13(5): 787-796, 1999.
13. Yee D, Jackson JG, Weng C-N, Gooch JL, Lee AV. The IGF system in breast cancer. In: K. Takano, Hizuka N, Takahashi S-I (ed.). Molecular Mechanisms to regulate the activities of insulin-like growth factors, pp. 319-325: Elsvier Science B.V., Amsterdam, 1998.
14. Gooch JL, Lee AV, Yee D. Interleukin-4 (IL-4) induces growth inhibition and apoptosis in human breast cancer cells. *Cancer Research.* 58:4199-4205, 1998.
15. Gooch JL, Lee AV, Christy B, Yee D. The role of insulin receptor substrate-1 (IRS-1) in insulin-like growth factor -I (IGF-I)- and interleukin-4 (IL-4)-mediated growth effects in human breast cancer cells. *Cancer Research*, Under revision, 2001.
16. Gooch JL, Tang Y, Garcia S, Abboud HE. Inhibition of calcineurin blocks renal hypertrophy associated with STZ-induced diabetes. In preparation, 2001.

17. Gooch, JL, Christy, B, Yee D. Stat6 is required for IL-4-indiced growth inhibition and apoptosis in breast cancer cells. Submitted. 2001.

Abstracts:

1. JM Gilroy, M Arar, G Gosh Choudhury, HE Abboud. Differntial Mitogenic Signaling of Platelet-derived Growth Factor (PDGF) Isoforms In Rat Metanephric Mesenchymal Cells (MMC): Activation of Phosphatidylinositol 3 Kinase (PI3K) and Mitogen Activated Protein Kinase (MAPK). Mol. Biol. Of Cell. Vol. 9, abstract 1820, 1998.
2. JM Gilroy, B Zhang, G Ghosh Choudhury, P Soriano, M Arrar, HE Abboud. Signaling of PDGF isoforms in PDGF β receptor deficient metanephric mesenchymal cells (MMC): PDGF α mediates migration but not DNA synthesis. J. Am. Soc. Nephrol., 10, Abstract 2039, p404A,1999.
3. G Ghosh Choudhury, JM Gilroy, HE Abboud. Synergistic Action of interfong (IFNg) on PDGF-induced DNA Synthesis is mediated via c-fos gene transcription in mesangial cells (MC). J. Am. Soc. Nephrol., 10, Abstract A2470, p489A,1999.
4. M Arar, M Gonzales, J Gilroy, M Bunegin, G Ghosh Choudhury, J Barnes, I Elshihabi, HE Abboud. Effect of Fibronectin in Tubulogenesis. J. Am. Soc. Nephrol., 10, Abstract A2026, p468A,1999.
5. JM Ricono, B-X Zhang, G Ghosh Choudhury, P Soriano, HE Abboud. Differential Effect of PDGF AA and BB on Migration of Metanephric Mesenchymal Cells (MMC): Role of Calcium. J. Am. Soc. Nephrol., 11, Abstract A1998, p318A, 2000.
6. JL Gooch, Yping Tang, JM Ricono, HE Abboud. Insulin-like growth factor-I induces renal hypertrophy via a calcineurin-dependent mechanism. J. Am. Soc. Nephrol., 11, Abstract A2396, 2000.
7. JM Ricono, Y Gorin, B-X Zhang, J-H Zhang, G Ghosh Choudhury, P Soriano and HEAbboud. Differential effect of PDGF AA and BB on migration of metanephric mesenchymal cells (MMCs): role of calcium and reactive oxygen species. Keystone symposium, Cell migration and invasion. Abstract 339, p82, 2001.
8. Gooch JL, Van Den Berg CL, Yee D. Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-mediated cell death: proliferative and anti-apoptotic effects (Abstract). 21st Annual San Antonio Breast Cancer Symposium, San Antonio, TX. *Breast Cancer Research and Treatment* 50: 273, 1998.
9. Gooch JL, Yee D. Interferon gamma signaling in breast cancer (Abstract). 20th Annual San Antonio Breast Cancer Symposium, San Antonio, TX. *Breast Cancer Research and Treatment* 46: 116, 1997.

Conclusions

The original investigators have reported that IL-4-mediated growth inhibition is associated with increased apoptosis and that IL-4 activates two important signaling molecules, IRS-1 and STAT6. Inhibition of IRS-1 does not block IL-4-mediated growth effects while inhibition of STAT6 decreases IL-4-mediated growth inhibition and apoptosis. Additionally, over-expression of STAT6 mimics the effect of IL-4. Finally, the investigators examined the mechanism of INF gamma-mediated growth inhibition of breast cancer cells in vitro. In addition to the JAK-STAT pathway, INF gamma decreases breast cancer cell growth in p21 dependent and independent pathways.

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References

Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U. 2001. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. Nat Cell Biol 3:512-516.

Heldin CH, Ernlund A, Rorsman C, Ronnstrand L. 1989. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. J Biol Chem 264:8905-8912.

Heldin CH, Westermark B. 1989. Platelet-derived growth factors: a family of isoforms that bind to two distinct receptors. Br Med Bull 45:453-464.

Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. 1994. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 8:1875-1887.

Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U. 2000. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. Nat Cell Biol 2:302-309.

Seifert RA, van Koppen A, Bowen-Pope DF. 1993. PDGF-AB requires PDGF receptor alpha-subunits for high-affinity, but not for low-affinity, binding and signal transduction. J Biol Chem 268:4473-4480.

Soriano P. 1994. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 8:1888-1896.

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Effect of platelet-derived growth factor isoforms in rat metanephric mesenchymal cells

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Departments of [1]Medicine and [3]Pediatrics, and [2]Institute of Biotechnology, Department of Molecular Medicine, [4]The University of Texas Health Science Center and Geriatric Research, Education and Clinical Center, [5]South Texas Veterans Health Care System, San Antonio, Texas 78229-3900

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Ricono, Jill M., Mazen Arar, Goutam Ghosh Choudhury, and Hanna E. Abboud. Effect of platelet-derived growth factor isoforms in rat metanephric mesenchymal cells. *Am J Physiol Renal Physiol* 282: F211–F219, 2002. First published August 8, 2001; 10.1152/ajprenal.00323.2000.—Platelet-derived growth factor (PDGF) B-chain or PDGF β-receptor-deficient mice lack mesangial cells. To explore potential mechanisms for failure of PDGF A-chain to rescue mesangial cell phenotype, we investigated the biological effects and signaling pathways of PDGF AA and PDGF BB in metanephric mesenchymal (MM) cells isolated from rat kidney. PDGF AA caused modest cell migration but had no effect on DNA synthesis, unlike PDGF BB, which potently stimulated migration and DNA synthesis. PDGF AA and PDGF BB significantly increased the activities of phosphatidylinositol 3-kinase (PI 3-K) and mitogen-activated protein kinase (MAPK). PDGF BB was more potent than PDGF AA in activating PI 3-K or MAPK in these cells. Pretreatment of MM cells with the MAPK kinase (MEK) inhibitor PD-098059 abrogated PDGF BB-induced DNA synthesis, whereas the PI 3-K inhibitor wortmannin had a very modest inhibitory effect on DNA synthesis (approximately Δ20%). On the other hand, wortmannin completely blocked PDGF AA- and PDGF BB-induced migration, whereas PD-098059 had a modest inhibitory effect on cell migration. These data demonstrate that activation of MAPK is necessary for the mitogenic effect of PDGF BB, whereas PI 3-K is required for the chemotactic effect of PDGF AA and PDGF BB. Although PDGF AA stimulates PI 3-K and MAPK activity, it is not mitogenic and only modestly chemotactic. Collectively, the data may have implications related to the failure of PDGF AA to rescue mesangial cell phenotype in PDGF B-chain or PDGF-β-receptor deficiency.

kidney; development; mesangial cell; signal transduction

THERE IS EVIDENCE THAT GLOMERULAR microvascular cells arise from metanephric mesenchyme (14). The spatial and temporal distribution of platelet-derived growth factor (PDGF) and its receptors (PDGFR) suggest a role for this growth factor in the development of mesangial cells (18) and have been conclusively demonstrated in two studies where PDGF B-chain or PDGFR-β-deficient mice lack mesangial cells (16, 21). PDGF is widely expressed in a variety of mesenchymal cells during development. Siefert et al. (18) mapped the expression patterns of PDGF ligands and receptors in the developing and adult murine kidney using in situ hybridization (Fig. 1). During glomerular development, as the renal vesicle epithelium progresses through the comma- and S-shape stages, PDGF A-chain and B-chains are expressed in epithelial cells. PDGF A-chain is expressed earlier and is seen even at the renal vesicle stage, whereas PDGF B-chain expression occurs at later stages and, at the earliest, is seen in the S-shaped bodies of the developing glomerular structures. PDGFR-α and PDGFR-β are expressed by mesenchymal cells in the metanephric blastema. At later stages, PDGFR-α is expressed in cells surrounding the glomerulus, and PDGFR-β transcripts are present in the mesenchymal/interstitial cells that are recruited into the glomerular cleft, which will form the vascular tuft of the mature glomerulus (18). These cells express PDGFR-β and PDGF B-chain transcripts at high levels during the later stages of glomerular development when microvasular (capillary) cells begin to fill the glomerular tuft. At this stage, PDGFR-α transcripts are barely detectable and PDGF A-chain transcripts are undetectable. Utilizing both immunohistochemistry and in situ hybridization, Arar et al. (2) showed similar findings in the rat, with PDGFR-β localizing to metanephric mesenchymal (MM) cells at early stages of development, cells within the cleft of the S-shaped bodies of the maturing glomerulus, and, at later stages, in the mature glomerulus. This study also demonstrated that activation of PDGFR-β by PDGF BB isoform mediates MM cell migration and DNA synthesis, providing one mechanism by which a subpopulation of these cells potentially develop into mesangial cells. However, the failure of PDGFR-α to compensate for the lack of β-receptor in the PDGFR-β-deficient mice remains unexplained, considering that the α-receptor, similar to the β-receptor, localizes to mesenchymal cells in the metanephric blastema, and

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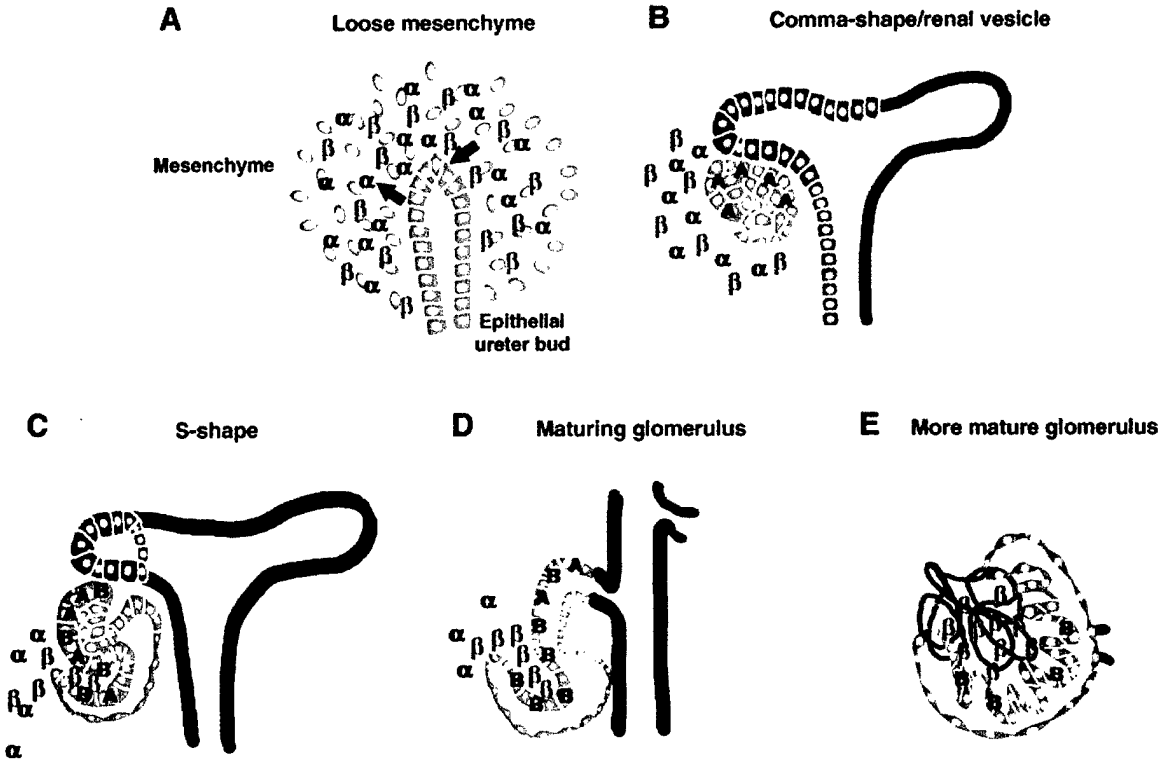

Fig. 1. Schematic representation of the localization of platelet-derived growth factor (PDGF) ligands and receptors at various stages of the developing kidney (*A–E*) based on data from Siefert et al. (18) and Arar et al. (2).

PDGF A-chain, similar to the B-chain, is also expressed in epithelial cells of the maturing glomerulus.

Binding of PDGF dimers to the extracellular domain of the receptor induces the receptor to dimerize and transautophosphorylate. Autophosphorylation of the receptor increases its intrinsic tyrosine kinase activity and provides docking sites for downstream signal transduction proteins (1, 9). Many of these tyrosines interact directly or indirectly with the SH2 domains of signaling molecules and are present in homologous positions with respect to PDGFR-α and PDGFR-β. Activation of PDGFR initiates several major signal transduction cascades, which include activation of phosphoinositiol 3-kinase (PI 3-K), phospholipase C (PLC)γ_1, and Ras-Raf-mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK- [extracellular signal-activated kinase (ERK)1/2] pathways (3, 5, 8). Expression of mutant PDGFR-β, the tyrosine residues of which are replaced with phenylalanine, demonstrated an essential role for PI 3-K and PLCγ_1 in proliferation and chemotaxis in different cell types (6, 20, 22, 23). Additional studies demonstrated that these responses are cell-specific (12). Because PDGF B-chain and PDGFR-β appear to be essential for mesangial cell development, and signaling through the PDGFR-α does not seem to compensate for the loss of PDGFR-β signaling, we examined the biological effects of PDGF AA and PDGF BB on MM cells and the role of ERK1/2 and PI 3-K.

MATERIALS AND METHODS

Materials. Tissue culture materials were purchased from GIBCO BRL (Rockville, MD). Recombinant PDGF-AA and PDGF-BB were obtained from R&D Systems (Minneapolis, MN). Wortmannin and PD-098059 were purchased from Alexis (San Diego, CA). Myelin basic protein (MBP), phosphatidylinositol (PI), collagenase, and collagen type I were obtained from Sigma (St. Louis, MO). Primary antibodies to PDGFR-β (A-3) and PDGFR-α (951) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated to Cy3 or FITC were obtained from Chemicon (Temecula, CA). Protein measurement and polyacrylamide gel reagents were purchased from Bio-Rad (Hercules, CA). Anti-phosphotyrosine and Erk-1 polyclonal antibodies were also purchased from Santa Cruz Biotechnology. Protein A-Sepharose was obtained from Pierce (Rockford, IL). All other reagents were high-quality analytic grade.

Cultured cells. Primary MM cell cultures were prepared as previously described (2, 10). Briefly, pregnant Sprague-Dawley rats were purchased at 10–11 days of gestation. At gestational *day 13*, mothers were anesthetized by intramuscular injection of rat mixture (60% ketamine, 40% xylazine), and embryos were collected. The age of the embryo was counted from the day of the vaginal plug (*day 0*). Embryos were dissected in 1× phosphate-buffered saline under a zoom model SZH Olympus stereomicroscope. Embryonic kidneys were collected, and cells were propagated in Dulbecco's modified Eagle medium (GIBCO) including 10% fetal calf serum and grown at 37°C, 5% carbon dioxide.

MAPK assay. MM cells were plated 7.5×10^5 cells/60-mm dish, grown to confluency, and serum starved for 48 h. Re-

spective cells were pretreated with 50 μM PD-098059, an MEK inhibitor, for 45 min before being stimulated with PDGF-AA or PDGF-BB. Cells were lysed with RIPA buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4 , 1 mM polymethylsulfonyl fluoride, and 0.1% aprotinin) for 30 min at 4°C and centrifuged at 10,000 *g* for 20 min at 4°C. Protein concentrations were measured in the cell lysates. Equal amounts of protein (100 μg) were incubated with ERK-1 polyclonal antibody for 30 min on ice. Fifteen microliters of protein A-Sepharose beads (50% vol/vol slurry) were added and incubated at 4°C on a rocking platform for 2 h. The immunobeads were washed and resuspended in MAPK assay buffer (in mM: 10 HEPES, pH 7.4, 10 $MgCl_2$, 0.5 dithiothreitol, and 0.5 Na_3VO_4) in the presence of 0.5 mg/ml MBP and 25 μM cold ATP plus 1 μCi [γ-^{32}P]ATP. The mixture was incubated at 30°C for 30 min, followed by a 10-min incubation on ice. Protein-loading buffer was added, and reactions were boiled. Samples were then loaded on a 12.5% SDS-PAGE, and phosphorylated MBP was visualized by autoradiography (5).

Western blotting. Equal amounts of protein from cell lysates were separated on a 12.5% SDS-PAGE gel and electrophoretically transferred to polyvinyl membrane. The membrane was blocked with 5% nonfat milk prepared in Tris-buffered saline with Tween 20 (TBST) buffer, washed with TBST, and incubated with ERK-1 primary antibody (1:200 dilution; Santa Cruz Biotechnology). The membrane was then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The blot was developed with enhanced chemiluminesence reagent.

PI 3-K assay. MM cells were plated as above. Respective cells were pretreated with 250 nM wortmannin, a PI 3-K inhibitor, for 3 h before treatment with PDGF. Cells were lysed and protein was analyzed as previously mentioned. One hundred micrograms of protein were incubated with anti-phoshotyrosine antibody for 30 min on ice. Fifteen microliters protein A-Sepharose beads (50% vol/vol slurry) were added and incubated at 4°C on a rocking platform for 2 h. The immunobeads were washed three times with RIPA, once with PBS, once with *buffer A* (0.5 mM LiCl, 0.1 M Tris·HCl, pH 7.5, and 1 mM Na_3VO_4), once with doubly distilled water, and once with *buffer B* (0.1 M NaCl, 0.5 mM EDTA, 20 mM Tris·HCl, pH 7.5). The immunobeads were then resuspended in 50 μl of PI 3-K assay buffer (20 mM Tris·HCl, pH 7.5, 0.1 M NaCl, and 0.5 mM EGTA). PI (0.5 μl of 20 mg/ml) was added and incubated at 25°C for 10 min. A cocktail of 1 μl of 1 M $MgCl_2$ and 10 μCi [γ-^{32}P]ATP was added and incubated at room temperature for 10 min. A mixture of chloroform, methanol, and 11.6 N HCl (150 μl, 50:100:1) was added to stop the reaction, and an additional 100 μl of chloroform were added. The organic layer is extracted and washed with methanol and 1 N HCl (1:1). The reaction was dried overnight and resuspended in 10 μl of chloroform. The samples were separated by thin-layer chromatography and developed with $CHCl_3$/MeOH/28% NH_4OH/H_2O (129:114:15:21). The spots were visualized by autoradiography (5).

DNA synthesis. MM cells were plated at 7.5×10^4 cells/24-well dish, grown to confluency, and serum starved for 48 h. Cells were either pretreated with PD-098059 or wortmannin as previously described before stimulation with PDGF isoforms. [^{3}H]TdR (1 μCi/25 ml) was added to each well. DNA synthesis is measured as the incorporation of [^{3}H]thymidine into trichoroactic acid-insoluble material (7).

Cell migration assay. Cell migration in response to PDGF was determined using blind well chamber assays. Confluent MM cells were serum starved for 48 h and then pretreated with PD-098059, wortmannin, or LY-294002. The monolayer of cells were trypsinized and resuspended in serum-free media. The cell suspension was added to the top chamber, while the PDGF was added to the bottom chamber of the apparatus. A polycarbonate membrane filter coated with collagen I separated the chambers. After 4 h at 37°C, the cells on the upper surface of the filter were removed with a cotton-tipped applicator, and migratory cells on the lower surface of the filter were fixed in methanol and stained with Giemsa. With the use of high magnification (×450), the migration of cells was analyzed by counting the number of cells that had migrated through the polycarbonate filter (7).

Immunofluorescence. For PDGFR-α and -β double immunofluorescent staining, cells were grown to near confluency on eight-well coverslips. Cells were fixed in methanol and washed in 1× PBS with 0.1% BSA. The primary antibody (PDGFR-β) 1:20 was added, and coverslips were incubated in a humidifier for 30 min at room temperature. Cells were washed three times for 5 min each. The respective secondary antibody (donkey anti-mouse, Cy3 conjugated) 1:30 was added, and coverslips were incubated in a humidifier for an additional 30 min at room temperature. For the PDGFR-α, cells were washed three times for 5 min each, the primary antibody (PDGFR-α) 1:20 was added, and coverslips were incubated in a humidifier for 30 min at room temperature. The respective secondary antibody (donkey anti-rabbit, FITC conjugated) 1:20 was added, and coverslips were incubated in a humidifier for an additional 30 min at room temperature. Cells were washed three times for 5 min each and mounted with crystal mounts. Cells were viewed with respective fluorescent filters with ultraviolet light.

RESULTS

Effect of PDGF isoforms on MM cell DNA synthesis and migration. PDGF AA and PDGF BB were examined for their ability to stimulate DNA synthesis as measured by [^{3}H]thymidine incorporation into DNA of quiescent MM cells. When cells were treated with PDGF BB for 24 h, [^{3}H]thymidine incorporation increased nearly fivefold above basal levels at concentrations of 10 and 100 ng/ml of PDGF BB. However, similar concentrations of PDGF AA did not increase DNA synthesis above basal levels (Fig. 2*A*). In addition to proliferation, migration is an important biological response during organ development. MM cells from the same passage used for the [^{3}H]thymidine incorporation were used for the migration assays. PDGF BB induced migration of MM cells four- to fivefold above baseline, with a maximal effect seen at a dose of 10 ng/ml. PDGF AA also induced migration in the MM cells about twofold above basal levels at a dose of both 10 and 100 ng/ml; however, the response was much weaker than that of PDGF BB (Fig. 2*B*).

PDGF activation of MAPK in MM cells. Activated PDGFR-β is known to associate with Grb2/sos, which lies upstream of the Ras-MEK-ERK signaling pathway. In contrast, activated PDGFR-α has been reported to associate with Crk adaptor protein, which may also lie upstream of the Ras-MEK-ERK signaling pathway. To determine whether MAPK (ERK1/2) is activated in MM cells, we measured the kinase activity in ERK1/2 immunoprecipitates of PDGF-stimulated MM cells. MM cells were stimulated with PDGF AA or PDGF BB for 5, 10, and 15 min. Both PDGF AA and

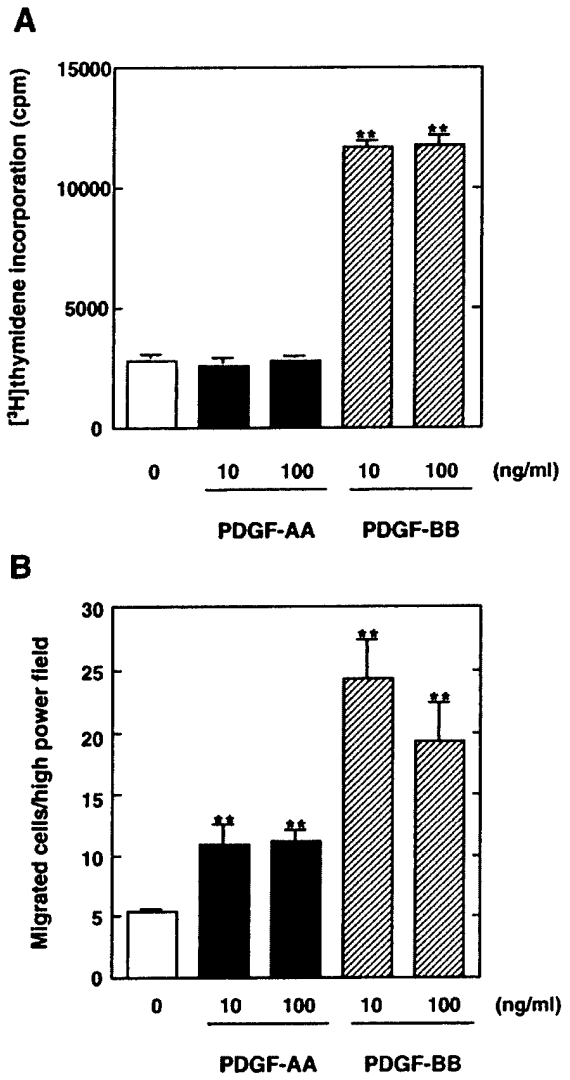

Fig. 2. Effect of PDGF isoforms on DNA synthesis and migration. *A*: [^{3}H]thymidine incorporation was measured as an index of DNA synthesis in response to treatment of 10 or 100 ng/ml of PDGF AA or PDGF BB in quiescent metanephric mesenchymal (MM) cells. Values are means ± SE of 4 independent experiments. $^{**}P < 0.01$ vs. untreated control. *B*: serum-deprived quiescent MM cells were used in cell migration assay in the presence of 10 or 100 ng/ml of PDGF AA or PDGF BB as described in MATERIALS AND METHODS. Values are means ± SE of 3 independent experiments. $^{**}P < 0.01$ vs. untreated control.

PDGF BB stimulated ERK1/2 activity in MM cells (Fig. 3). Maximal activation was observed at 15 min, and therefore in all subsequent experiments cells were treated for 15 min with the PDGF isoforms. Dose-responses of PDGF-induced ERK1/2 activity showed that PDGF AA induced maximal ERK1/2 activity at 50 and 100 ng/ml, whereas PDGF BB induced maximum activity at 10 ng/ml (Fig. 4). These concentrations correspond to those required to stimulate proliferation and migration (Fig. 2, *A* and *B*). In some experiments, cells were pretreated with an MEK inhibitor, PD-098059, and assayed for ERK1/2 activity (Fig. 5). PD-098059 reduced PDGF-induced ERK1/2 activity to near basal levels, indicating that ERK1/2 activation in MM cells is mediated by the Ras-Raf-MEK-MAPK pathway. Western blot analysis of the ERK1/2 protein was performed on the same cell lysates as for loading controls.

Effect of MAPK inhibitor on PDGF-induced DNA synthesis and migration in MM cells. To assess the involvement of MAPK signaling pathways in DNA synthesis and migration of the MM cells, cells were pretreated with 50 μM PD-098095 for 45 min before the addition of PDGF AA or PDGF BB. Pretreatment of cells with the MEK inhibitor abolished PDGF-induced DNA synthesis (Fig. 6*A*). This suggests that the Ras-Raf-MEK-MAPK pathway is a major contributor of PDGF-induced DNA synthesis in MM cells. Pretreatment of the MM cells with PD-098095 significantly

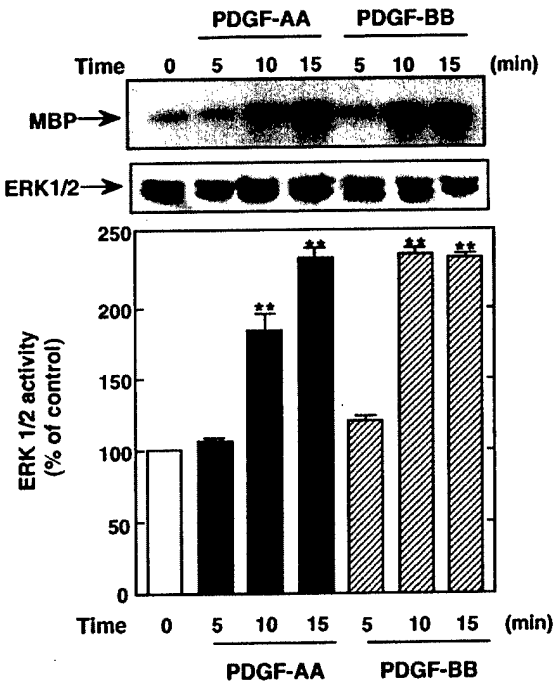

Fig. 3. Time course of activation of mitogen-activated protein kinase (MAPK) in PDGF-treated MM cells. Serum-deprived quiescent MM cells were treated with 100 ng/ml of PDGF AA or 10 ng/ml of PDGF BB for various time points, i.e., 5, 10, and 15 min. Cleared cell lysates were immunoprecipitated with extracellular signal-regulated kinase (ERK)-1 polyclonal antibody. The immunoprecipitates were then used in an in vitro immunecomplex kinase assay in the presence of myelin basic protein (MBP) and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. Phosphorylated MBP was separated on a 12.5% SDS-polyacrylamide gel. Western blot analysis of ERK 1/2 was done on the same cell lysates to determine the loading control. Each barogram represents the ratio of the radioactivity incorporated into the phosphorylated MBP quantified by PhosphorImager analysis factored by the densitometric measurement of the ERK1/2 band. Values are means ± SE of 3 independent experiments expressed as the percentage of control, where the ratio in the untreated cells was defined as 100%. $^{**}P < 0.01$ vs. control.

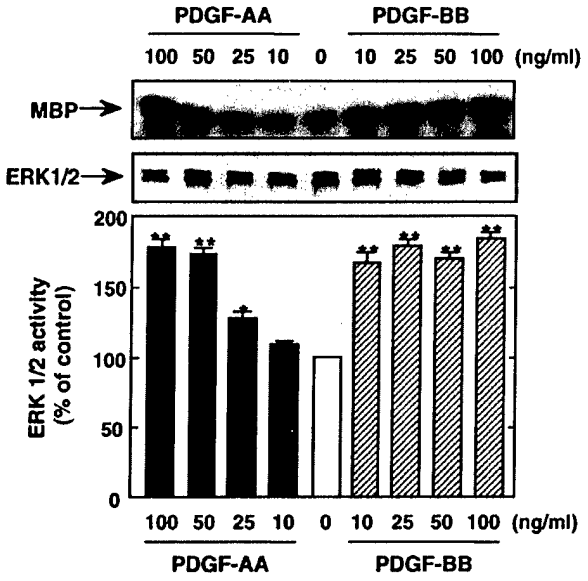

Fig. 4. Effect of different doses of PDGF AA and BB on MAPK activity in MM cells. Quiescent MM cells were treated with varying concentrations of PDGF AA and BB. Cells were stimulated for 15 min with 10, 25, 50, or 100 ng/ml of PDGF AA or BB. Cleared cell lysates were immunoprecipitated with ERK-1 polyclonal antibody. The immunoprecipitates were then used in an in vitro immunecomplex kinase assay in the presence of MBP and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. Phosphorylated MBP was separated on a polyacrylamide gel. Western blot analysis of ERK 1/2 was done on the same cell lysates, which were used as loading controls. Each barogram represents the ratio of the radioactivity incorporated into the phosphorylated MBP, quantified by PhosphorImager analysis factored by the densitometric measurement of ERK1/2 band. Values are mean ± SE of 3 independent experiments and are expressed as the percentage of control, where the ratio in the untreated cells was defined as 100%. $^{**}P < 0.01$ vs. control. $^{*}P < 0.05$.

decreased migration induced by PDGF BB (Fig. 6*B*). However, PD-098095 did not inhibit migration to basal levels as in PDGF-induced DNA synthesis (Fig. 6*A*). The same concentration of PD-098095 that completely blocked PDGF AA-induced ERK1/2 activity (Fig. 5) did not significantly reduce PDGF AA-induced migration. These data suggest that other pathways are involved in PDGF-induced migration.

PDGF activation of PI 3-K. PI 3-K has previously been shown to associate with tyrosine-phosphorylated PDGF receptors. However, it has not been established whether signaling by both PDGF isoforms through their respective receptors can activate PI 3-K in MM cells. PI 3-K activity was determined in anti-phosphotyrosine immunoprecipitates of lysate from PDGF AA- or PDGF BB-stimulated cells. The immunoprecipitates were assayed for PI 3-K activity as described in MATERIALS AND METHODS. As shown in Fig. 7, both isoforms of PDGF activate PI 3-K activity. PDGF BB showed a significant effect at 10 ng/ml, whereas 100 ng/ml of PDGF AA was necessary to induce significant activation. When cells were pretreated with wortmaninn, a PI 3-K inhibitor, the PDGFR-associated PI 3-K activity in MM cells was significantly reduced (Fig. 8).

Effect of PI 3-K inhibitor on PDGF-induced DNA synthesis and migration in MM cells. We have recently shown that activation of PI 3-K is necessary for PDGF-induced DNA synthesis and migration in mesangial cells (4). To determine the importance of PI 3-K in MM cells, cells were pretreated with 250 nM wortmannin for 3 h before stimulation with PDGF isoforms. Wortmannin decreased PDGF-induced [^{3}H]thymidine incorporation by ~20% (Fig. 9*A*), unlike the complete abolition of activity when cells were pretreated with PD-098059 (Fig. 6*A*). These data indicate that PI 3-K plays a lesser role in PDGF-induced DNA synthesis than MAPK in MM cells. Two structurally dissimilar PI 3-K inhibitors, wortmannin and LY-294002, completely blocked PDGF-induced migration in MM cells (Fig. 9, *B* and *C*), whereas the MEK inhibitor decreased PDGF BB-induced migration by ~30% and PDGF AA-induced migration by ~15% (Fig. 6*B*). These data suggest that PI 3-K, rather than MAPK, plays a predominant role in PDGF-induced migration.

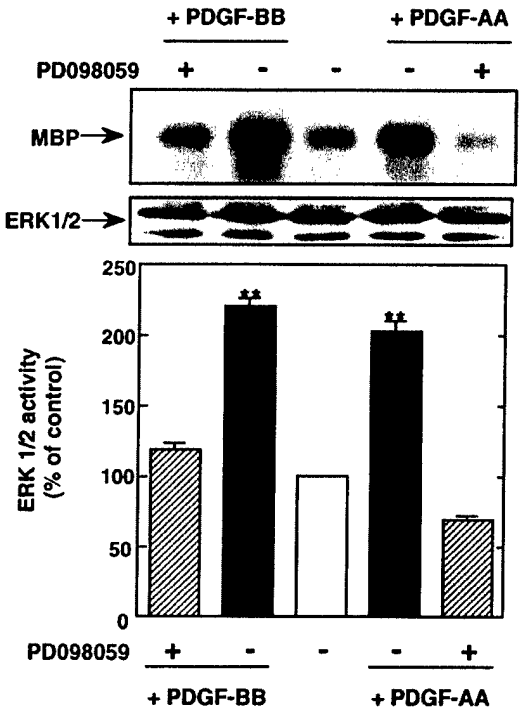

Fig. 5. Effect of MEK inhibitor on the Ras-Raf-MAPK kinase (MEK)-ERK pathway. Quiescent MM cells were pretreated with 50 μM PD-098059 for 45 min before treatment with 100 ng/ml of PDGF AA or 10 ng/ml of PDGF BB. Cleared cell lysates were immunoprecipitated with ERK-1 polyclonal antibody. The immunoprecipitates were then used in an in vitro immunecomplex kinase assay in the presence of MBP and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. Phosphorylated MBP was separated on a 12.5% SDS-polyacrylamide gel. Western blot analysis of ERK1/2 was done on the same cell lysates and used as loading controls. Each barogram represents the ratio of the radioactivity incorporated into the phosphorylated myelin basic protein quantified by PhosphorImager analysis factored by the densitometric measurement of ERK1/2 band. Values are means ± SE of 3 independent experiments and are expressed as the percentage of control, where the ratio in the untreated cells was defined as 100%. $^{**}P < 0.01$ vs. control.

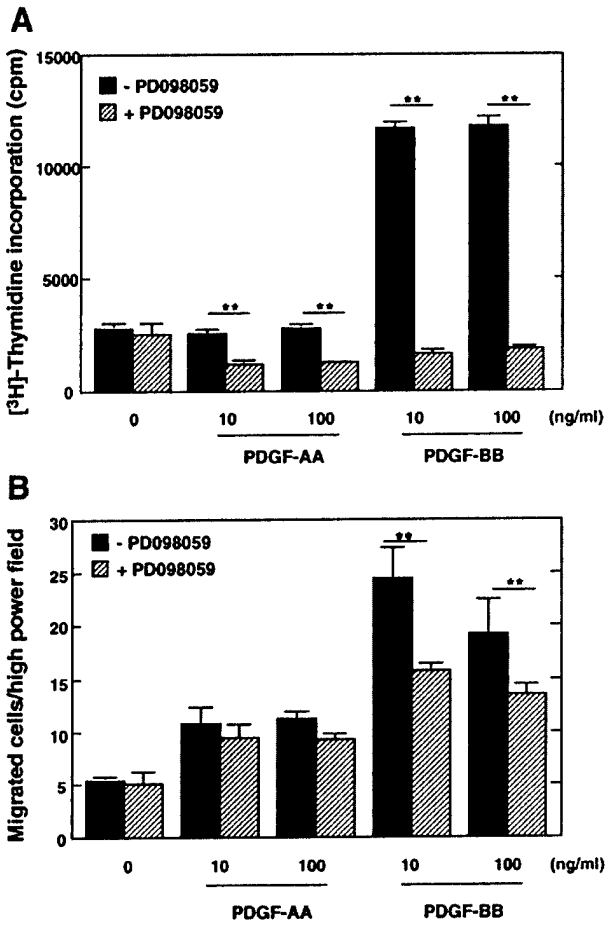

Fig. 6. Effect of MEK inhibitor on PDGF-induced DNA synthesis and migration. Quiescent MM cells were pretreated with 50 μM PD-098059 for 45 min before treatment with PDGF AA or PDGF BB. *A*: [^{3}H]thymidine incorporation was measured as an index of DNA synthesis in response to treatment of 10 or 100 ng/ml of PDGF AA or BB in quiescent MM cells. Values are means ± SE of 4 independent experiments. $^{**}P < 0.01$ vs. untreated and treated with PD-098059. *B*: serum-deprived quiescent MM cells were used in cell migration assay in the presence of 10 or 100 ng/ml of PDGF AA or BB, as described in MATERIALS AND METHODS. Values are means ± SE of 3 independent experiments. $^{**}P < 0.01$ vs. untreated and treated with PD-098059.

Expression of PDGF receptors in MM cells. To study the expression of PDGFR-α and -β in MM cells, immunofluorescence was performed using PDGFR-α- and PDGFR-β-specific antibodies. Figure 10 shows abundant expression of both PDGFR-α and PDGFR-β in MM cells. This suggests that these cells have the potential to respond to both PDGF isoforms.

DISCUSSION

There are several potential mechanisms by which PDGF, acting on MM cells, leads to the development of a subset of these cells into mature differentiated mesangial cell phenotype. Two mechanisms pertinent to development include cell migration and cell proliferation. This study explored the effects of PDGF AA and BB isoforms on DNA synthesis and migration of MM cells isolated at the earliest stage of the developing rat metanephric blastema, embryonic *day 13*. Arar et al. (2) have recently demonstrated that PDGF BB stimulates DNA synthesis and migration of these cells. Stimulation of cell migration by PDGF BB was associated with activation of PI 3-K, and inhibition of PI 3-K blocked PDGF BB-induced migration. However, the role of other signal transduction pathways activated by

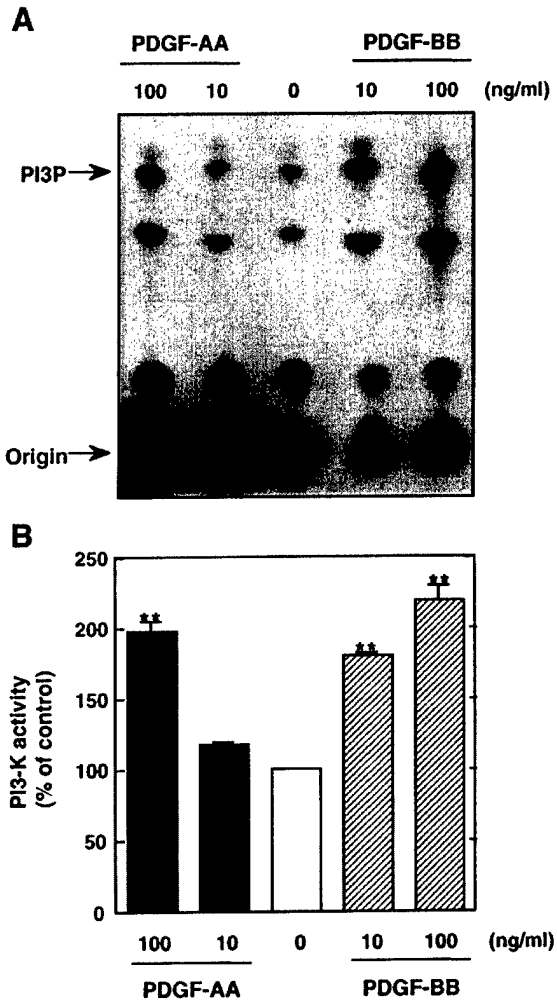

Fig. 7. Activation of phosphatidylinositol 3-kinase (PI 3-K) in PDGF-treated MM cells. *A*: serum-deprived MM cells were treated with 10 or 100 ng/ml PDGF AA or PDGF BB for 15 min and cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were then assayed for PI 3-kinase activity in the presence of phosphotidyl inositol (PI) and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. The arrow indicates the position of PI 3-P spot. *B*: each barogram represents the radioactivity incorporated into PI 3-P by PhosphorImager analysis. Values are means ± SE of 3 independent experiments and are expressed as the percentage of control, where the untreated cells were defined as 100%. $^{**}P < 0.01$ vs. untreated control.

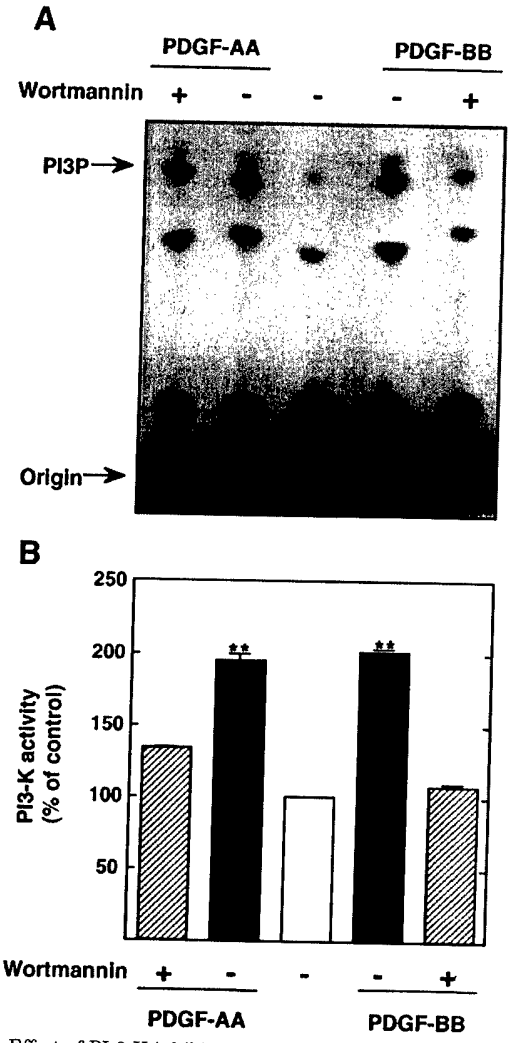

Fig. 8. Effect of PI 3-K inhibitor on PDGF-activated PI 3-K in MM cells. *A*: quiescent MM cells were pretreated with 250 nM wortmannin for 3 h before treatment with PDGF AA or PDGF BB. Cleared cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and the immunoprecipitates were then assayed for PI 3-K activity in the presence of PI and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. Arrow, position of PI 3-P spot. *B*: each barogram represents the radioactivity incorporated into PI 3-P by PhosphorImager analysis. Values are means ± SE of 3 independent experiments and are expressed as the percentage of control, where the untreated cells were defined as 100%. $^{**}P < 0.01$ vs. untreated and treated with wortmannin.

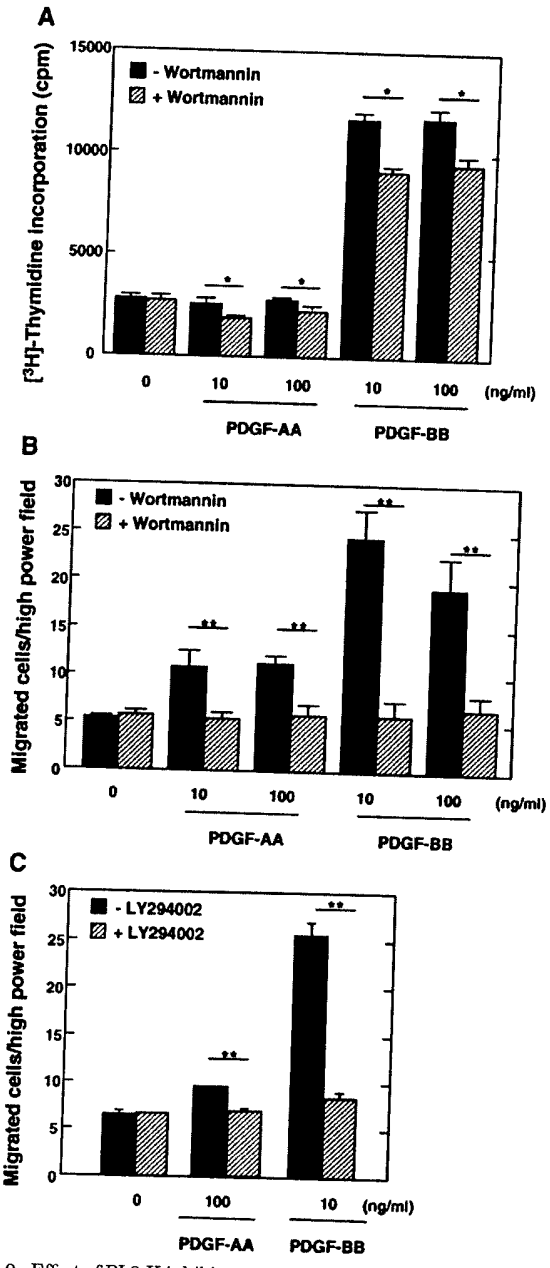

Fig. 9. Effect of PI 3-K inhibitor on PDGF-induced DNA synthesis and migration. Quiescent MM cells were pretreated with 250 nM wortmannin for 3 h or 25 μM LY-294002 for 1 h before treatment with PDGF AA or PDGF BB. *A*: [^{3}H]thymidine incorporation was measured as an index of DNA synthesis in response to treatment of 10 or 100 ng/ml of PDGF AA or BB in quiescent MM cells. Results are means ± SE of 4 independent experiments. $^{*}P < 0.05$ vs. untreated and treated with wortmannin. *B*: serum-deprived quiescent MM cells were used in cell migration assay in the presence of 10 or 100 ng/ml of PDGF AA or PDGF BB, as described in MATERIALS AND METHODS. The data represent means ± SE of 3 independent experiments. $^{**}P < 0.01$ vs. untreated and treated with wortmannin. *C*: serum-deprived quiescent MM cells were used in cell migration assay in the presence of 100 ng/ml of PDGF AA or 10 ng/ml of PDGF BB, as described in MATERIALS AND METHODS. The data represent means ± SE of 3 independent experiments. $^{**}P < 0.01$ vs. untreated and treated with LY-294002.

PDGF BB was not explored. More importantly, the role of the PDGF A-chain in activating MM cells is not known. This information is pertinent because PDGFR-α does not appear to compensate for PDGFR-β in rescuing mesangial cell phenotype in PDGFR-β deficiency. Moreover, during early stages of development and maturation of the glomerular capillary bed, the PDGF A-chain and PDGFR-α have a spatial and temporal distribution similar to that of the PDGF B-chain and PDGFR-β, respectively (18).

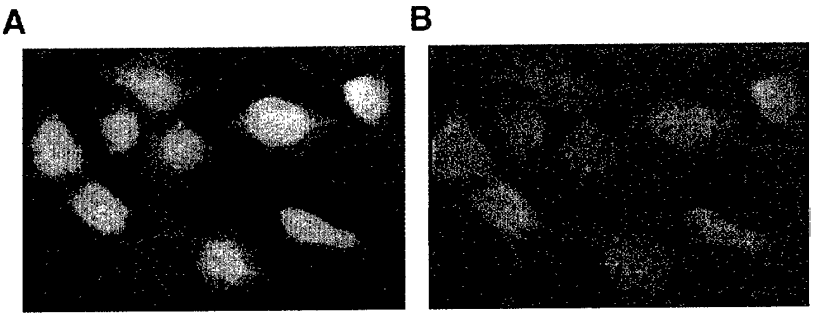

Fig. 10. Expression of PDGFR-α and -β in MM cells. *A*: immunofluorescent localization of PDGFR-α. *B*: immunofluorescent localization of PDGFR-β. All MM cells stained for both PDGFR-α and -β. Magnification: ×40.

PDGF BB, as reported previously (2), stimulates DNA synthesis and robust migration of MM cells. We now demonstrate that PDGF AA, even at a concentration as high as 100 ng/ml, was not mitogenic for these cells and had a modest effect on cell migration. Therefore, the lack of a mitogenic effect of the PDGF A-chain and its inability to stimulate robust migration are potential mechanisms for failure of PDGFR-α to compensate for PDGFR-β in rescuing mesangial cell phenotype. Mouse chimeras composed of PDGFR-β +/+ and PDGFR-β −/− cells demonstrated that PDGFR β −/− cells fail to populate the glomerular mesangium, whereas PDGFR β +/+ cells do, suggesting a direct permissive role of PDGF BB in mesangial cell development and maturation (17). Studies utilizing bromodeoxyuridine labeling demonstrated active proliferation of mesangial cell progenitors in cup-shaped and S-shaped glomeruli of wild-type, but not mutant, mice. These studies suggested that proliferation of mesangial cell progenitors is a critical step for mesangial cell development (17). Our finding of the failure of PDGF AA to induce proliferation of MM cells supports this contention. We next examined the activation of ERK1/2 by PDGF AA or PDGF BB and its involvement in PDGF-induced DNA synthesis and migration of MM cells. PDGF AA and PDGF BB increased ERK1/2 activity in a dose- and time-dependent manner (Figs. 3 and 4), with PDGF BB being slightly more potent than PDGF AA. Pretreatment of MM cells with the MEK inhibitor PD-098059 at a concentration that abolished MAPK activity resulted in complete inhibition of DNA synthesis. However, the MEK inhibitor only partially blocked PDGF BB-induced cell migration and exerted a small but insignificant effect on PDGF AA-induced cell migration. These data indicate that the Ras-Raf-MEK-MAPK pathway is essential for PDGF BB-induced DNA synthesis in MM cells.

Cells expressing a PDGFR-β mutant devoid of the binding sites for PI 3-K, i.e., lacking Tyr^{740} and Tyr^{751}, show no chemotactic responses to PDGF (15, 24), suggesting a role for PI 3-K in cell migration. However, PI 3-K regulates growth factor-induced migration in a cell type-specific manner. For example, there is evidence that PI 3-K does not mediate cell migration in smooth muscle cells activated by PDGF BB (12). PDGFR-α-mediated migration also appears to be cell type specific. In lung fibroblasts, Swiss 3T3 and hematopoeitic 32D cells, activation of PDGFR-α induces migration (13, 19, 25). However, in other cell types, such as aortic endothelial cells and vascular smooth muscle cells, PDGF AA inhibits the chemotactic response. PDGF BB as well as PDGF AA induce PI 3-K activity in MM cells, with the AA isoform resulting in somewhat lesser induction of enzyme activity. Wortmannin, at concentrations that decreased PI 3-K enzymatic activity, markedly inhibited PDGF-induced migration of MM cells. In contrast to its potent effect on cell migration, pretreatment of MM cells with wortmannin reduced PDGF BB-induced DNA synthesis by ~20%. The data indicate that migration of MM cells in response to both PDGF isoforms is mediated via PI 3-K. It is very unlikely that the differential effect of PDGF isoforms is due to differential expression of PDGFR-α and -β in the cells, because both receptors were homogenously distributed in MM cells. The data also demonstrate that the lack of biological response to PDGF AA is not due to a low number of PDGFR-α or poor coupling of the AA ligand with the receptor, because PDGF AA was almost as potent as PDGF BB in activating ERK1/2 at a wide range of concentrations. Furthermore, the PDGF AA isoform potently activated PI 3-K to a degree almost similar to that for the PDGF BB isoform. However, activation of these pathways, PI 3-K and MAPK, by PDGF AA is not sufficient to induce DNA synthesis or robust migration in these cells. These data, taken together with the mesangial cell phenotype in the PDGFR-β-deficient mouse, suggest that, in the absence of significant DNA synthesis, activation of PI 3-K and subsequent migration is insufficient for PDGFR-α, which can be activated by PDGF AA or PDGF BB, to compensate for the loss of PDGFR-β. Of interest is the recent observation that mice with a PDGFR-β mutant for PI 3-K binding sites develop normally and do not exhibit an overt phenotype in the mesangium (11), suggesting that β-receptor-mediated signaling through activated PI 3-K is only of minor importance during mesangial cell development. Alternatively, other signaling molecules activated by the mutant β-receptor are able to compensate for the loss of PI 3-K signaling.

In conclusion, in this study we have shown that PDGF AA and PDGF BB activate PI 3-K and MAPK enzymatic signaling pathways in MM cells. We have also shown that PDGF BB induces DNA synthesis primarily through the MAPK pathway and migration

through the PI 3-K pathway. The finding that PDGF AA had no effect on DNA synthesis, whereas it stimulated modest migration of the cells, suggests that the failure of PDGFR-α to compensate for loss of PDGFR-β may be due to its inability to mediate these fundamental biological responses of MM cells. It is tempting to speculate that mesangial cell progenitors that may be stimulated to migrate eventually undergo apoptosis in the absence of the PDGF B-chain or PDGFR-β or fail to sustain their proliferation or even their survival. A more comprehensive examination of molecules activated by PDGFR-β is required to understand the mechanism by which PDGF BB and PDGFR-β activation result in mesangial cell development and maturation.

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REFERENCES

1. **Abboud H, Bhandari B, and Choudhury GG.** Cell biology of platelet-derived growth factor. In: *Molecular Nephrology. Kidney Function in Health and Disease*, edited by Bonventre J and Schlondorf D. New York: Dekker, 1995, p. 573–590.
2. **Arar M, Xu YC, Elshihabi I, Barnes JL, Choudhury GG, and Abboud HE.** Platelet-derived growth factor receptor beta regulates migration and DNA synthesis in metanephric mesenchymal cells. *J Biol Chem* 275: 9527–9533, 2000.
3. **Bazenet CE and Kazlauskas A.** The PDGF receptor alpha subunit activates p21ras and triggers DNA synthesis without interacting with rasGAP. *Oncogene* 9: 517–525, 1994.
4. **Choudhury GG, Grandaliano G, Jin DC, Katz MS, and Abboud HE.** Activation of PLC and PI 3 kinase by PDGF receptor alpha is not sufficient for mitogenesis and migration in mesangial cells. *Kidney Int* 57: 908–917, 2000.
5. **Choudhury GG, Karamitsos C, Hernandez J, Gentilini A, Bardgette J, and Abboud HE.** PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF. *Am J Physiol Renal Physiol* 273: F931–F938, 1997.
6. **Cospedal R, Abedi H, and Zachary I.** Platelet-derived growth factor-BB (PDGF-BB) regulation of migration and focal adhesion kinase phosphorylation in rabbit aortic vascular smooth muscle cells: roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinases. *Cardiovasc Res* 41: 708–721, 1999.
7. **Grandaliano G, Valente AJ, Rozek MM, and Abboud HE.** Gamma interferon stimulates monocyte chemotactic protein (MCP-1) in human mesangial cells. *J Lab Clin Med* 123: 282–289, 1994.
8. **Heldin CH.** Structural and functional studies on platelet-derived growth factor. *Embo J* 11: 4251–4259, 1992.
9. **Heldin CH, Ostman A, and Ronnstrand L.** Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1378: 79–113, 1998.
10. **Herzlinger D, Koseki C, Mikawa T, and al-Awqati Q.** Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development* 114: 565–572, 1992.
11. **Heuchel R, Berg A, Tallquist M, Ahlen K, Reed RK, Rubin K, Claesson-Welsh L, Heldin CH, and Soriano P.** Platelet-derived growth factor beta receptor regulates interstitial fluid homeostasis through phosphatidylinositol-3′ kinase signaling. *Proc Natl Acad Sci USA* 96: 11410–11415, 1999.
12. **Higaki M, Sakaue H, Ogawa W, Kasuga M, and Shimokado K.** Phosphatidylinositol 3-kinase-independent signal transduction pathway for platelet-derived growth factor-induced chemotaxis. *J Biol Chem* 271: 29342–29346, 1996.
13. **Hosang M, Rouge M, Wipf B, Eggimann B, Kaufmann F, and Hunziker W.** Both homodimeric isoforms of PDGF (AA and BB) have mitogenic and chemotactic activity and stimulate phosphoinositol turnover. *J Cell Physiol* 140: 558–564, 1989.
14. **Hyink DP, Tucker DC, St John PL, Leardkamolkarn V, Accavitti MA, Abrass CK, and Abrahamson DR.** Endogenous origin of glomerular endothelial and mesangial cells in grafts of embryonic kidneys. *Am J Physiol Renal Fluid Electrolyte Physiol* 270: F886–F899, 1996.
15. **Kundra V, Escobedo JA, Kazlauskas A, Kim HK, Rhee SG, Williams LT, and Zetter BR.** Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. *Nature* 367: 474–476, 1994.
16. **Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, and Betsholtz C.** Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8: 1875–1887, 1994.
17. **Lindahl P, Hellstrom M, Kalen M, Karlsson L, Pekny M, Pekna M, Soriano P, and Betsholtz C.** Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development* 125: 3313–3322, 1998.
18. **Seifert RA, Alpers CE, and Bowen-Pope DF.** Expression of platelet-derived growth factor and its receptors in the developing and adult mouse kidney. *Kidney Int* 54: 731–746, 1998.
19. **Siegbahn A, Hammacher A, Westermark B, and Heldin CH.** Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J Clin Invest* 85: 916–920, 1990.
20. **Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, and Bleasdale JE.** Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J Pharmacol Exp Ther* 253: 688–697, 1990.
21. **Soriano P.** Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8: 1888–1896, 1994.
22. **Valius M, Bazenet C, and Kazlauskas A.** Tyrosines 1021 and 1009 are phosphorylation sites in the carboxy terminus of the platelet-derived growth factor receptor beta subunit and are required for binding of phospholipase C gamma and a 64-kilodalton protein, respectively. *Mol Cell Biol* 13: 133–143, 1993.
23. **Valius M, Secrist JP, and Kazlauskas A.** The GTPase-activating protein of Ras suppresses platelet-derived growth factor beta receptor signaling by silencing phospholipase C-gamma 1. *Mol Cell Biol* 15: 3058–3071, 1995.
24. **Wennstrom S, Siegbahn A, Yokote K, Arvidsson AK, Heldin CH, Mori S, and Claesson-Welsh L.** Membrane ruffling and chemotaxis transduced by the PDGF beta-receptor require the binding site for phosphatidylinositol 3′ kinase. *Oncogene* 9: 651–660, 1994.
25. **Yu JC, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, and Aaronson SA.** Tyrosine mutations within the alpha platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol 3-kinase activity without affecting mitogenic or chemotactic signal transduction. *Mol Cell Biol* 11: 3780–3785, 1991.

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Insulin-like Growth Factor-I Induces Renal Cell Hypertrophy via a Calcineurin-dependent Mechanism*

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Insulin-like growth factor-I (IGF-I) may play an important role in the development of renal hypertrophy. In this study we determined the effect of IGF-I on cultured mesangial cells (MCs) and examined activation of key signaling pathways. IGF-I induced hypertrophy as determined by an increase in cell size and an increase in protein to DNA ratio and increased accumulation of extracellular matrix (ECM) proteins. IGF-I also activated both Erk1/Erk2 MAPK and phosphatidylinositol 3-kinase (PI3K) in MCs. Inhibition of either MAPK or PI3K, however, had no effect on IGF-I-induced hypertrophy or ECM production. Next, we examined the effect of IGF-I on activation of the calcium-dependent phosphatase calcineurin. IGF-I treatment stimulated calcineurin activity and increased the protein levels of calcineurin and the calcineurin binding protein, calmodulin. Cyclosporin A, an inhibitor of calcineurin, blocked both IGF-I-mediated hypertrophy and up-regulation of ECM. In addition, calcineurin resulted in sustained Akt activation, indicating possible cross-talk with other signaling pathways. Finally, IGF-I treatment resulted in the calcineurindependent nuclear localization of NFATc1. Therefore, IGF-I induces hypertrophy and increases ECM accumulation in MCs. IGF-I-mediated hypertrophy is associated with activation of Erk1/Erk2 MAPK and PI3K but does not require either of these pathways. Instead, IGF-I mediates hypertrophy via a calcineurin-dependent pathway.

In response to stress or injury, kidney tissue undergoes hypertrophy, and to a lesser extent hyperplasia, resulting in a net gain in the size of the kidney. Glomeruli, the filtering microvascular structures, are particularly susceptible to hypertrophy, which eventuates in fibrosis. At the cellular level, hypertrophy is characterized by cessation of the cell cycle at G_1, a halt in DNA synthesis, and continued production and/or decreased degradation of cellular proteins (1). The net result is an increase in protein concentration disproportionate to DNA and an increase in the overall size of the cell. In addition to the increase in cell size, expansion of the extracellular matrix (ECM),[1] including fibronectin and collagen type IV, contributes to tissue hypertrophy. Studies in humans and in animal models of renal hypertrophy indicate that early hypertrophy and ECM accumulation are potentially reversible (2). Therefore, understanding the mechanisms that are required for the induction and maintenance of hypertrophy and ECM accumulation by growth factors, hormones, and cytokines may be critical for developing therapies that prevent or reverse renal hypertrophy.

The insulin-like growth factor (IGF) system has been implicated in glomerular hypertrophy. In patients with type I diabetes, elevated amounts of IGF-I in the urine are associated with hypertrophy and progression of kidney disease (3). Moreover, endogenous kidney IGF-I levels are elevated within 2–3 days of streptozotocin-induced type I diabetes in rats (4, 5) and IGF-I receptor is up-regulated after prolonged hyperglycemia (6). Although overexpression of growth hormone (which regulates IGF-I) causes both hypertrophy and sclerosis of mouse kidneys (7), increased circulating levels of IGF-I result primarily in hypertrophy (8). This suggests that endogenous rather than circulating levels of IGF-I mediate hypertrophy and subsequent sclerosis. Alternatively, IGF-I is necessary for hypertrophy but may be insufficient for development of sclerosis. Flyvbjerg *et al.* (9) found that treatment of diabetic rats with octreotide, a somatostatin inhibitor, decreased circulating IGF-I and IGF-II levels. Moreover, kidney hypertrophy was decreased and urinary albumin excretion was blocked (9). Several other studies support this finding (2, 10). Although IGF-I is undoubtedly an important growth factor that mediates vascular renal pathology, very little is known about the signaling pathways activated by IGF-I in renal cells.

In some cells, IGF-I commonly signals through two main pathways, the phosphatidylinositol 3-kinase (PI3K) and Erk1/Erk2 MAPK pathways (11). Both are activated following association of insulin receptor substrate (IRS) proteins with the IGF-I receptor and coupling of the activated tyrosine kinase receptor to downstream signaling targets. However, there is very little evidence regarding what pathway(s) IGF-I activates in specific renal cells, including those derived from the glomerulus. di Mari *et al.* (12) showed that IGF-I treatment results in activation of Erk1/Erk2 MAPK in proximal tubule cells. In addition, Horney *et al.* (13) showed phosphorylation of the adaptor proteins IRS1 and IRS2 by IGF-I in cultured mesangial cells. Mesangial cells constitute one third of glomerular cell populations and contribute to hypertrophy of glomeruli seen in

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[1] The abbreviations used are: ECM, extracellular matrix; DN, diabetic nephropathy; IGF-I, insulin-like growth factor-I; MCs, mesangial cell; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; SFM, serum-free medium; NFAT, nuclear factor of activated T cells; ERK, extracellular signal-regulated kinase; IRS, insulin receptor substrate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting.

diabetes or other conditions characterized by renal hypertrophy (2, 4).

In this study, we examined the effect of IGF-I on cultured MCs from glomeruli of rats and evaluated both proliferative and hypertrophic effects. We also examined the signaling pathways activated by IGF-I, including the MAPK and PI3K pathways. In addition, we investigated IGF-I-mediated activation of other pathways, including the calcium-dependent serine/threonine phosphatase, calcineurin. Accordingly, we determined what effect inhibition of these pathways has on IGF-I-mediated hypertrophy and ECM production. Finally, we demonstrated the significance of calcineurin activation by showing IGF-I-mediated nuclear translocation of the calcineurin substrate, nuclear factor of activated T cells- c1 (NFATc1).

EXPERIMENTAL PROCEDURES

Materials

Receptor grade recombinant human IGF-I was purchased from GroPep (Adelaide, Australia), and recombinant human TGFβ1 was obtained from R&D Systems, Inc. (Minneapolis, MN). PD98059 was from Calbiochem (La Jolla, CA). Wortmannin, cyclosporin A, calcium ionophore A23187, and anti-fibronectin antibody were from Sigma Chemical Co. (St. Louis, MO). Anti-collagen type IV antibody was purchased from Chemicon (Temecula, CA), anti-phospho Akt, anti-phospho-Erk1/Erk2, and anti-calcineurin antibodies were from Transduction Laboratories (San Diego, CA), and anti-NFATc1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Rat MCs were cultured from glomeruli isolated by differential sieving as previously described (14). Epithelial cells were removed by digestion with collagenase, and glomerular cores were cultured in RPMI (Life Technologies, Inc., Gaithersburg, MD) supplemented with antibiotics and 17% fetal calf serum. For these experiments, rat MCs that have been maintained in our laboratory (University of Texas Health Science Center, San Antonio, TX) were used between passage 26 and 32.

Hypertrophy

FACS—MCs were plated in 60-mm plates and allowed to grow to 80–90% confluency. Medium was then changed to serum-free media (SFM) for 24 h, and the cells were treated as indicated. After treatment, MCs were harvested by trypsinization, washed with 1× PBS, centrifuged at 5000 rpm for 2 min, and then resuspended in ice-cold 70% ethanol added dropwise while vortexing. Ethanol-fixed MCs were then analyzed by forward light scattering on a Becton Dickinson flow cytometer.

Protein/DNA Ratio—MCs were plated in triplicate in 12-well plates and allowed to grow to 80–90% confluence. Medium was changed to SFM for 24 h, and the cells were treated as indicated. Each well was washed, collected by trypsinization, and split into two equal aliquots. Both samples were washed with 1× PBS and centrifuged at 5000 rpm for 2 min. One aliquot was resuspended in TNESV protein lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotinin, and 10^{-7} M phenylmethylsulfonyl (PMSF)) and the other in ice-cold 70% ethanol. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). For determination of DNA concentration, Hoescht stain (Sigma, St. Louis, MO) was added to ethanol fixed cells for 30 min at room temperature. DNA was measured at 355-nm excitation and 460-nm emission using a Titertak Flouroskan II fluorometer. For each well the resulting protein determination was divided by the DNA measurement to provide a protein/DNA ratio.

Kinase Assay

MCs were plated in 60-mm plates and allowed to grow to 80–90% confluency (~2 days). Medium was then changed to SFM for 24 h, and the cells were treated as indicated.

Erk1/Erk2 MAPK—Erk1/Erk2 was immunoprecipitated from 100 μg of total protein using anti-Erk1 antibody (Santa Cruz Biotechnology) followed by protein A-Sepharose beads. Immunocomplexes were washed three times in TNESV buffer and resuspended in kinase buffer (10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM Na_3VO_4) in the presence of 0.5 mg/ml myelin basic protein (MBP), 25 μM cold ATP, and 1 μCi of [γ-^{32}P]ATP. Reactions were incubated for 30 min at 30 °C followed by 10-min incubation on ice. Phosphorylated MBP was resolved on 12% SDS-PAGE, the gel was dried, and an exposure was made onto film.

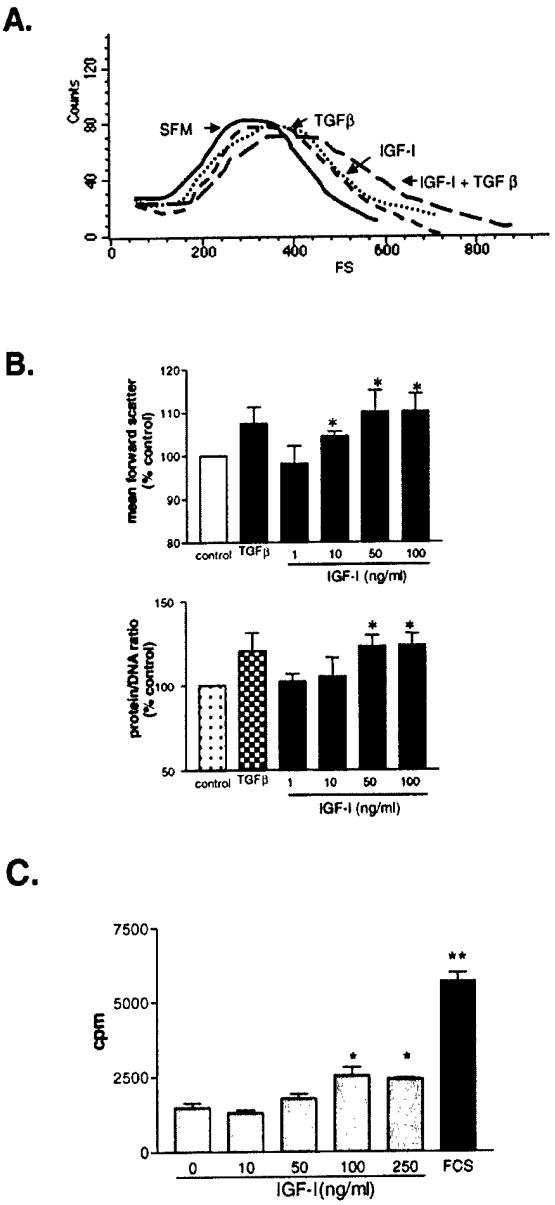

FIG. 1. **IGF-I induces hypertrophy of mesangial cells.** Near-confluent mesangial cells were serum-starved for 24 h and then treated as indicated: *A*, cells were treated with SFM (control), TGFβ (1 ng/ml), or IGF-I (50 ng/ml) for 72 h. Cell size was determined by FACS analysis using forward light scattering. *B*, cells were treated with SFM (control), TGFβ (1 ng/ml), or increasing amounts of IGF-I for 48 h, and then cell size was determined by forward light scattering. *Bars* represent the mean ± S.E. of four independent experiments. Cells were also analyzed for DNA and protein content, and the protein/DNA ratio of triplicate samples ± S.E. was determined. Data shown are representative of at least three independent experiments. *, $p < 0.05$ compared with control. *C*, cells were treated for 24 h with increasing amounts of IGF-I, and then DNA synthesis was measured with ^{3}H-labeled thymidine. Fetal calf serum (*FCS*) was included as a positive control for DNA synthesis. *Bars* represent the mean of triplicate samples ± S.E. Data shown are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.001$ compared with control.

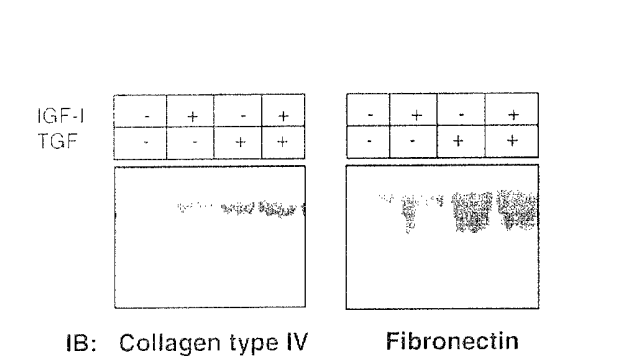

FIG. 2. **IGF-I treatment increases expression of collagen type IV and fibronectin proteins.** Near-confluent cells were serum-starved for 24 h and then treated with SFM alone (control), IGF-I (100 ng/ml), TGFβ (1 ng/ml), or IGF-I + TGFβ for 72 h. *A*, total cellular proteins were collected and separated by SDS-PAGE. Collagen type IV and fibronectin were detected by direct immunoblotting. *B*, cells were fixed in methanol, and fibronectin and collagen type IV were visualized using anti-fibronectin antibody followed by a Texas Red-conjugated anti-rabbit secondary antibody (*a–d*), and collagen type IV antibody followed by a fluorescein-conjugated anti-goat secondary antibody (*e–h*). *a* and *e*, SFM (control); *b* and *f*, IGF-I; *c* and *g*, TGFβ; *d* and *h*, IGF-1 plus TGFβ.

PI3K—Kinase activity was determined as described previously (16).[2] Briefly, tyrosine-phosphorylated proteins were immunoprecipitated from 100 μg of total protein samples by incubation with anti-phosphotyrosine antibody for 30 min on ice followed by addition of protein A-Sepharose beads and additional incubation on ice for 2 h. Immunocomplexes were washed three times with lysis buffer, once with PBS, once with buffer A (0.5 mM LiCl, 0.1 M Tris-HCl, pH. 7.5, 1 mM Na_3VO_4), once with double-distilled water, and once with buffer B (0.1 M NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH 7.5). The immunocomplexes were then resuspended in 50 μl of PI3K buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.5 mM EGTA). 0.5 μl of 20 mg/ml PI was added and incubated at 25 °C for 10 min. A mixture of 1 μl of 1 M $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP was added, and the mixture was incubated at room temperature for 10 min. 150 μl of a mixture of chloroform, methanol, and 11.6 N HCl (50:100:1, v/v) was added to stop the reaction, and an additional 100 μl of chloroform was added. The organic layer was extracted and washed with methanol and 1 N HCl (1:1). The reaction was dried overnight and resuspended in 10 μl of chloroform. The samples were separated by thin-layer chromatography and developed with $CHCl_3$/MeOH/28% NH_4OH/H_2O (129:114:15:21). The spots were visualized by autoradiography.

Western Blots

MCs were plated in 60-mm dishes and allowed to grow to 80–90% confluency (~ 2 days). Medium was then changed to SFM for 24 h, and the cells were treated as indicated. Cells were then harvested with trypsin-EDTA, pelleted, and washed with 1× PBS. Protein was extracted using TNESV lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotinin, and 10^{-3} M phenylmethylsulfonyl (PMSF)). 25 μg of protein was analyzed by 7.5% SDS-PAGE. Following transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk-TBST and then immunoblotted with 1:2000 dilution of anti-fibronectin or 1:1000 dilution of anti-collagen type IV antibody. Horseradish peroxidase-conjugated donkey-anti-rabbit or horseradish peroxidase-conjugated donkey-anti-goat secondary antibody was added at 1:2000 for fibronectin and collagen type IV, respectively, and proteins were visualized by enhanced chemiluminescence (Pierce).

Immunoflorescence

MCs were plated in 8-well chamber slides and allowed to grow to 80–90% confluency (~ 2 days). Medium was then changed to SFM for 24 h, and the cells were treated as indicated. Following treatment, the cells were fixed and permeabilized in 100% methanol for 5 min and then rehydrated in PBS-0.1% BSA for 15 min. Cells were then blocked with the appropriate IgG for 15 min, and then primary antibodies were added for 30 min. Anti-fibronectin, anti-collagen type IV, and anti-NFATc1 were all added at a concentration of 15 μg. Cells were washed three times for 5 min with PBS-0.1% BSA before addition of secondary antibodies as indicated. All secondary antibodies were used at a dilution of 1–100. Finally, cells were washed three times for 5 min with PBS-0.1% BSA, the chambers were removed from the slides, and the coverslips were mounted with Crystal Mount (Biomeda, Foster City, CA). Cells were viewed by fluorescence microscopy.

Calcineurin Phosphatase Assay

Calcineurin phosphatase activity was determined following a protocol published by Fruman *et al.* (17). Briefly, the RII peptide was phosphorylated *in vitro* with 250 units of recombinant PKA, 50 mM ATP, 50 μCi of [γ-^{32}P]ATP, 0.15 mM RII, and 500 μl of 2× reaction buffer (40 mM MOPS, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.4 mM EDTA, 0.8 mM EGTA, 0.5 mM DTT, 0.1 mg/ml BSA). Lysates were prepared by resuspending cells in a hypotonic lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 50 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) followed by 3 cycles of freeze thawing in liquid nitrogen and a 30 °C water bath. Calcineurin activity in each sample was determined by incubating equal parts lysate, 3× reaction buffer (40 mM Tris, pH 7.5, 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM DTT, 500 nM okadaic acid, and 0.1 mg/ml BSA), and labeled RII peptide at 30 °C for 10 min. The reaction was stopped by addition of 0.1 M KPO_4 in 5% trichloroacetic acid. To determine the amount of phosphate released by calcineurin in each sample, reactions were then added to PolyPrep columns (Bio-Rad, Hercules, CA) containing AG-50X Dowex ion exchange resin (Bio-Rad) prepared as described in a previous study (17). Finally, 5 ml of scintillation fluid was added to the flow-through from each column, and the released phosphate was measured in a scintilla-

[2] J. M. Ricono, M. Arar, G. Gosh Choudhury, and H. E. Abboud (2001) *Am. J. Physiol.*, in press.

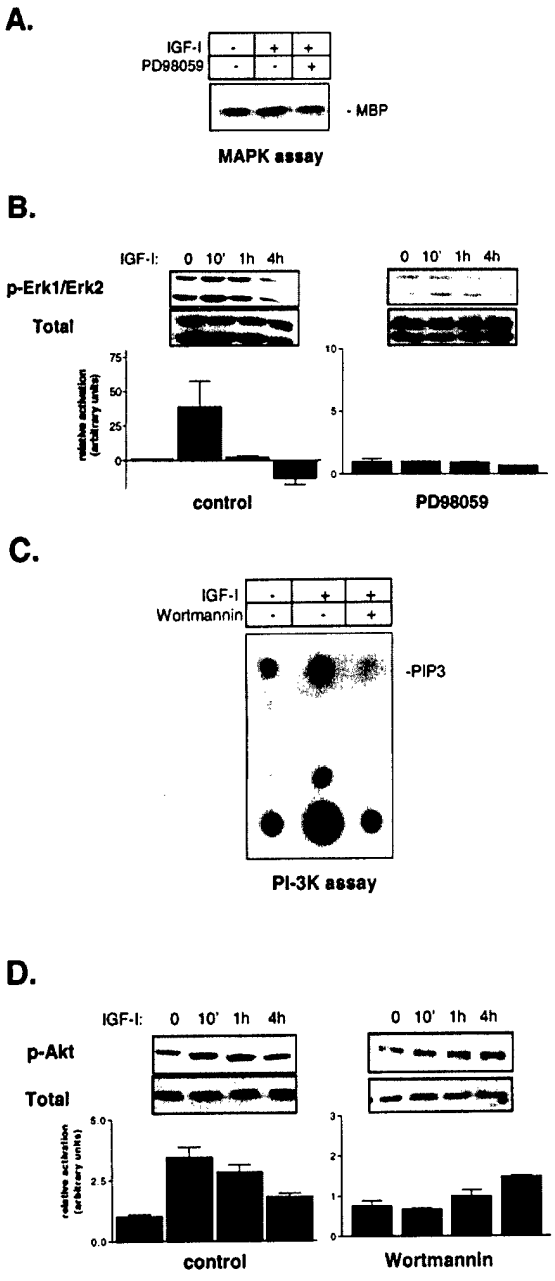

FIG. 3. **IGF-I-mediated hypertrophy is associated with activation of Erk1/Erk2 MAPK and PI3K pathways.** *A*, cells were pretreated with PD98059 (10 μM) and then incubated with IGF-I for 10 min. Erk1/Erk2 MAPK kinase activity was determined by *in vitro* kinase assay with MBP as a substrate. *B*, cells were pretreated with SFM (control) or PD98059 (10 μM) and then treated with IGF-I (50 ng/ml) for the indicated lengths of time. Protein lysates were collected, and phosphorylation of Erk1/Erk2 was determined by immunoblotting with phospho-specific Erk1/Erk2 antibody. Total Erk1/Erk2 was also detected in the same samples using a specific antibody. Relative activation from three separate experiments was quantitated and graphed. *C*, MCs were pretreated with wortmannin (250 nM) and then incubated with IGF-I for 10 min. PI3K activity was determined as described under "Experimental Procedures." *D*, cells were pretreated with SFM (control) or wortmannin (250 nM) and then treated with IGF-I (50 ng/ml) for the indicated lengths of time. Protein lysates were collected, and phosphorylation of Akt was determined by immunoblotting with a phospho-specific Akt antibody. Also, total Akt was detected in the same samples using a specific antibody. Relative activation from three separate experiments was quantitated and graphed.

tion counter. To ensure specificity of the assay for calcineurin activity, okadaic acid was routinely added to the reaction buffer to inhibit any dephosphorylation by serine-threonine phosphatases PP1A and PP2A.

Statistics

Statistical significance was determined by Student's *t* test. A result was considered significant if $p < 0.05$.

RESULTS

In vivo studies demonstrated that IGF-I plays a role in renal hypertrophy (4, 9, 18). However, it is unclear whether IGF-I acts as a proliferative agent for renal cells as it does with many other cells or if IGF-I can directly initiate hypertrophy. Therefore, in this study we examined the effect of IGF-I on cultured mesangial cells (MCs). Hypertrophy was determined as an increase in cell size (without an increase in cell number) and an increase in protein production without an increase in DNA synthesis. Fig. 1*A* shows that IGF-I treatment (50 ng/ml) of MCs resulted in an increase in cell size as determined by FACS analysis. The effect of IGF-I was comparable to the effect of TGFβ (1 ng/ml), which is known to induce hypertrophy in these cells (19). Treatment of MCs with both IGF-I and TGFβ resulted in an additive hypertrophic effect. FACS analysis of these cells showed that there was no difference in the distribution of cells in the cell cycle after IGF-I or TGFβ treatments compared with SFM control (data not shown).

IGF-I induced hypertrophy in a dose-dependent manner, with 50 ng/ml resulting in a significant increase in both cell size and protein/DNA ratio (Fig. 1*B*). The maximal effect of IGF-I was equal to or slightly greater than was seen with TGFβ treatment of these cells. Hypertrophy was discernible as early as 8 h after treatment and was detectable for as long as 72 h after exposure of cells to IGF-I (data not shown). Finally, there was a small increase in DNA synthesis following IGF-I treatment but only at concentrations of 100 ng/ml or greater (Fig. 1*C*).

There are conflicting reports regarding the ability of IGF-I to induce ECM production in renal cells. Previous *in vivo* experiments have shown that overexpression of IGF-I contributes to hypertrophy but is not sufficient for ECM production and development of fibrosis (7). However, other *in vitro* studies have shown that IGF-I does contribute to an increase in ECM (20, 21). In our experiments, MCs were treated with IGF-I or TGFβ for 72 h, and the levels of collagen type IV and fibronectin were determined by both Western blotting (Fig. 2*A*) and immunohistochemistry (Fig. 2*B*). We found that IGF-I induces an increase in both collagen type IV and fibronectin protein levels although not as robustly as does TGFβ. Interestingly, the amount of IGF-I that was sufficient to stimulate an increase in ECM (at least 100 ng/ml) was double that which was required to induce maximal hypertrophy (50 ng/ml). Similar to hypertrophy, IGF-I and TGFβ together resulted in an additive effect on the levels of ECM proteins.

We next examined the signaling pathways activated by IGF-I in MCs. Using an *in vitro* kinase assay with myelin basic protein (MBP) as a substrate for Erk1/Erk2 MAPK, we show that IGF-I treatment results in activation of MAPK in MCs (Fig. 3*A*). This activation was blocked by pretreatment with PD98059, an inhibitor of the Erk1/Erk2 kinase MEK. In addition, we treated MCs with IGF-I for increasing lengths of time and examined phosphorylation of Erk1/Erk2 using a phospho-specific Erk1/Erk2 antibody. Erk1/Erk2 was transiently phosphorylated following addition of IGF-I with maximal activation at 10 min (or less) that was reduced, even to below basal conditions, by 4 h. Pretreatment with PD98059 also blocked phosphorylation of Erk1/Erk2. Total Erk1/Erk2 protein level was determined by direct immunoblotting, and no change in the amount of Erk1/Erk2 protein was found (Fig. 3*B*). Next we

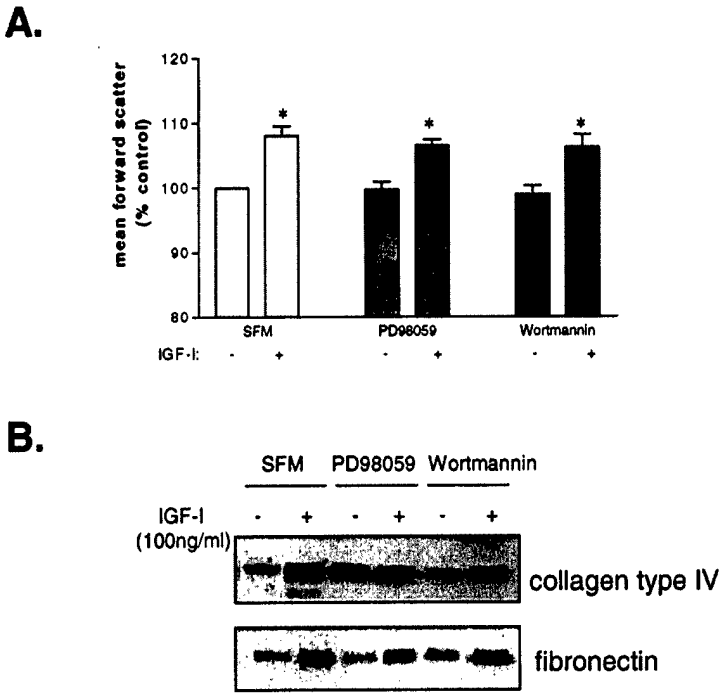

FIG. 4. **Inhibition of Erk1/Erk2 and PI3K pathways has no effect on induction of hypertrophy by IGF-I.** *A*, cells were pretreated with SFM, PD98059 (10 μM), or wortmannin (250 nM) for 1 h prior to the addition of IGF-I (50 ng/ml) for 24 h, and then cell sizes were determined by mean forward light scattering. *Bars* represent the mean of three independent experiments ± S.E. *, $p < 0.05$ compared with control. *B*, cells were pretreated with SFM, PD98059 (10 μM), or wortmannin (250 nM) for 1 h prior to the addition of IGF-I (100 ng/ml) for 72 h. Total cellular proteins were collected and separated by SDS-PAGE. Collagen type IV and fibronectin were detected by direct immunoblotting. Data shown are representative of at least three independent experiments.

examined activation of the PI3K pathway following IGF-I treatment in MCs. Fig. 3*C* shows that IGF-I activated PI3K and resulted in increased production of phosphatidylinositol 3-phosphate. This activation was completely blocked by wortmannin, an inhibitor of PI3K activity. We also treated MCs with IGF-I for increasing lengths of time and examined phosphorylation of Akt, a serine/threonine kinase regulated downstream of PI3K (Fig. 3*D*). Similar to Erk1/Erk2 phosphorylation, Akt phosphorylation was maximal at 10 min and decreased thereafter. Interestingly, although Akt phosphorylation peaked early, persistent activation was seen at 4 h. In addition, pretreatment with wortmannin blocked only IGF-I-mediated phosphorylation of Akt at 10 min. At 4 h, IGF-I-mediated phosphorylation of Akt was unaffected by wortmannin pretreatment. This suggests a PI3K-independent mechanism of sustained Akt phosphorylation. There were no changes in the levels of Akt protein.

Our experiments thus far indicate that IGF-I-mediated hypertrophy in MCs is associated with activation of Erk1/Erk2 MAPK and PI3K. Therefore, we next determined whether IGF-I-mediated hypertrophy was dependent on activation of either of these pathways. MCs were pretreated with inhibitors of Erk1/Erk2 MAPK (10 μM PD98059), PI3K (250 nM wortmannin), or vehicle alone (Me_2SO), and then IGF-I-mediated hypertrophy was assessed by FACS analysis. Fig. 4*A* shows that inhibition of Erk1/Erk2 MAPK or PI3K had no effect on IGF-I-induced increase in cell size. Similar results were obtained when protein/DNA ratio was measured. Also, similar results were obtained when PI3K was inhibited with the compound LY294002 (data not shown). We also determined the role of Erk1/Erk2 MAPK and PI3K signaling on IGF-I-mediated increase in ECM proteins collagen type IV and fibronectin. Fig. 4*B* shows that inhibition of either pathway failed to block the increase in ECM proteins by IGF-I.

Several studies have identified the calcium-regulated serine/threonine phosphatase calcineurin as a potential mediator of hypertrophy (22, 23). Therefore, we examined the role of calcineurin in IGF-I-mediated hypertrophy of MCs. Calcineurin phosphatase activity was determined in response to IGF-I using a highly specific *in vitro* phosphatase assay (17). The addition of IGF-I to the cells resulted in approximately a 50% increase in calcineurin activity (Fig. 5*A*). Calcineurin is inhibited by the immunosuppressive compound cyclosporin A. Cyclosporin A enters cells and binds to a group of proteins known as cyclophilins. Cyclosporin A-cyclophilin complexes, in turn, bind to the β subunit of calcineurin, resulting in inhibition of phosphatase activity (24, 25). Due to the specificity of action of cyclosporin A in inhibiting calcineurin, we used this compound to block IGF-I-mediated activation of calcineurin. Calcineurin phosphatase activity was determined in cells pretreated with cyclosporin A (5 μM), PD98059 (10 μM), or wortmannin (250 nM) and then stimulated with IGF-I for 2 h. As expected, pretreatment with cyclosporin A completely blocked calcineurin phosphatase activity, whereas inhibition of either Erk1/Erk2 MAPK or PI3K had no effect (Fig. 5*A*). We next examined the time course of calcineurin phosphatase activation by IGF-I. Fig. 5*B* shows that addition of IGF-I resulted in significant activation of calcineurin phosphatase within minutes of treatment that returned to basal levels after 4–6 h. Interestingly, phosphatase activity peaked roughly 2 h after IGF-I treatment, in contrast to the very early maximal phosphorylation of both Erk1/Erk2 MAPK and Akt. Increased calcineurin phosphatase activity has been shown to precede up-regulation of calcineurin protein (26). Accordingly, there was a substantial increase in calcineurin protein within 24 h of IGF-I treatment (Fig. 5*C*). Calcineurin activity is dependent upon binding both calcium and calmodulin. Therefore, we examined the effect of IGF-I on calmodulin protein levels. There was also a substantial increase in the calcineurin binding protein calmodulin in a time frame similar to the increase in calcineurin protein (Fig. 5*C*). In contrast, protein levels of Erk1/Erk2 MAPK did not change over the course of the experiment, indicating a selective increase in calcineurin/calmodulin proteins rather than a global increase in total proteins.

To determine if activation of calcineurin is required for IGF-I-induced hypertrophy, MCs were treated with cyclosporin A to

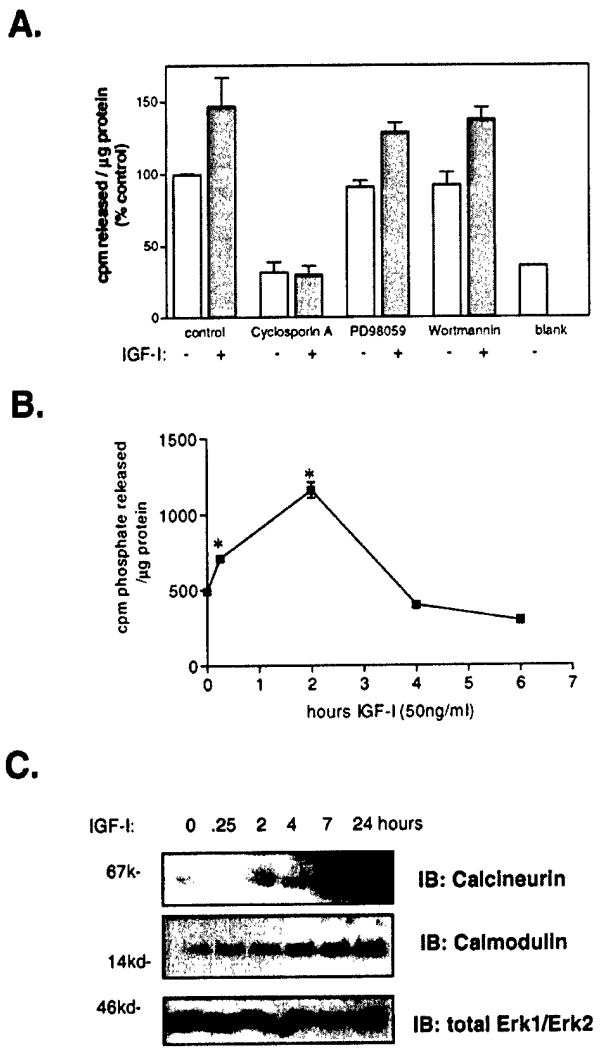

FIG. 5. **IGF-I stimulates calcineurin phosphatase activity and increases calcineurin and calmodulin proteins.** *A*, cells were pretreated with SFM, cyclosporin A (5 μM), PD98059 (10 μM), or wortmannin (250 nM) and then stimulated with IGF-I (50 ng/ml) for 2 h. Protein lysates were collected, and calcineurin phosphatase activity was determined as described under "Experimental Procedures." Data shown are the mean of duplicate assays ± S.E. *B*, calcineruin phosphatase activity was measured determined following treatment with IGF-I for up to 6 h. * $p < 0.01$ compared with 0 h treatment. Data shown are the mean of duplicate assays ± S.E. *C*, cells were treated with IGF-I (50 ng/ml) for up to 24 h and total protein lysates were collected. Calcineurin and calmodulin were detected by direct immunoblotting with specific antibodies. Data shown are representative of at least 3 independent experiments.

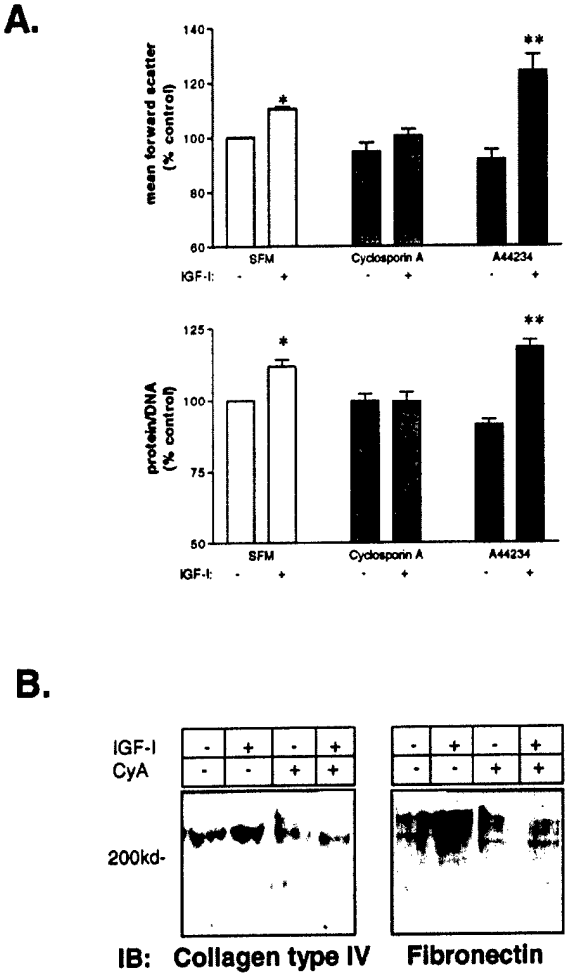

FIG. 6. **Calcineurin is required for IGF-I-mediated hypertrophy and up-regulation of ECM.** *A*, cells were pretreated for 1 h with SFM or cyclosporin A (5 μm) and then incubated with IGF-I for 24 h. Additionally, cells were pretreated with calcium ionophore A23187 (1.5 μm) for 1 h, washed twice with SFM, and then incubated with IGF-I for 24 h. Cells size was determined by mean forward light scatter. Bars represent the mean ± S.E. of four independent experiments. Cells were also analyzed for DNA and protein content and the protein/DNA ratio of triplicate samples ± S.E. was determined. * $p < 0.05$ from control and ** $p < 0.05$ from IGF-I. *B*, cells were pretreated for 1 h with SFM or cyclosporin A (5 μm) and then incubated with IGF-I (100 ng/ml) for 72 h. Total cellular proteins were collected and separated by SDS-PAGE. Collagen type IV and fibronectin were detected by direct immunoblotting. Data shown are representative of at least 3 independent experiments.

inhibit calcineurin activity prior to addition of IGF-I. Both IGF-I-mediated increase in cell size and increase in protein/DNA ratio were blocked following inhibition of calcineurin. Moreover, pretreatment of the cells with calcium ionophore A23187 to increase intracellular calcium significantly increased IGF-I-mediated hypertrophy (Fig. 6*A*). Next we determined if calcineurin was required for IGF-I-mediated induction of ECM. Fig. 6*B* shows that pretreatment with cyclosporin A blocked IGF-I-mediated increase in both collagen type IV and fibronectin protein levels. Cyclosporin A alone had no effect on either total protein synthesis (reflected by protein/DNA ratio) or on basal levels of collagen type IV or fibronectin.

Inhibition of PI3K with wortmannin only abolished Akt phosphorylation at 10 min and had no effect on IGF-I-mediated Akt phosphorylation at 4 h (Fig. 3*D*). The lack of inhibition of the delayed Akt activation by wortmannin suggests a PI3K-independent mechanism of sustained Akt activation. Therefore, in Fig. 7*A*, we examined the role of calcineurin in sustained Akt activation. MC were pretreated with SFM (control), wortmannin (250 nM), or cyclosporin A (5 μm) and then treated with IGF-I for 10 min or 4 h. Phosphorylated Akt was detected by Western blotting with a phospho-specific Akt antibody. At 10 min, wortmannin greatly reduced IGF-I-mediated Akt phosphorylation, as shown before, whereas cyclosporin A had no effect. After 4 h of IGF-I treatment, Akt phosphorylation is still detectable in control cells, and wortmannin pretreatment does not reduce this phosphorylation. However, pretreatment with

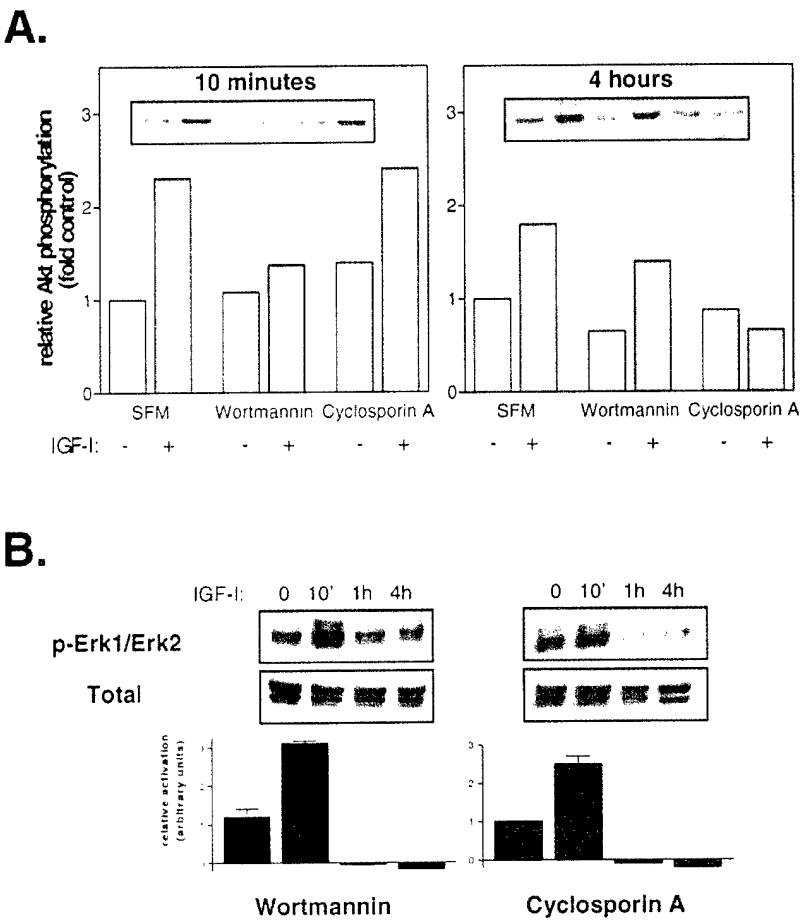

FIG. 7. **IGF-I induces sustained activation of Akt that is mediated by calcineurin.** *A*, cells were pretreated with SFM (control), wortmannin (250 nM), or cyclosporin A (5 μM) and then incubated with IGF-I for 10 min or 4 h. Total protein lysates were collected, and phosphorylated Akt was detected using a phospho-specific antibody. Relative Akt activation was determined by quantitation of phosphorylated Akt normalized by total Akt in each sample. *B*, cells were pretreated with PD98059 (10 μM) or cyclosporin A (5 μM) and then incubated with IGF-I for up to 4 h. Total protein lysates were collected, and phosphorylated Erk1/Erk2 was detected using a phospho-specific antibody.

cyclosporin A blocks IGF-I-mediated activation of Akt at 4 h. As a control, phospho-Erk1/Erk2 was also detected in cells pretreated with either wortmannin or cyclosporin A. Fig. 7*B* shows that inhibition of PI3K and calcineurin had no effect on Erk1/Erk2 phosphorylation. In addition, total Erk1/Erk2 protein levels did not change over the same time period.

One family of transcription factors that has been well characterized as downstream targets and mediators of calcineurin signaling is the Nuclear Factor of Activated T-cell (NFAT) family. These transcription factors are inactive in the cytosol and shuttle to the nucleus following dephosphorylation by calcineurin. NFATs associate with other transcription co-factors, including members of the Jun and Fos families and mediate transcription of target genes (27, 28). NFATc1–4 have all been shown to be regulated by calcineurin-mediated dephosphorylation. In addition, nuclear localization of NFATc1 has been shown to be associated with hypertrophy (22, 34). In Fig. 8 we show that 30 min of IGF-I treatment results in accumulation of NFATc1 in the nucleus. This nuclear localization of NFATc1 is dependent on calcineurin activation, because pretreatment with cyclosporin A blocks this effect entirely.

FIG. 8. **IGF-I treatment results in calcineurin-dependent nuclear localization of NFATc1.** Cells were serum-starved for 24 h and then incubated with SFM alone (control), IGF-I (50 ng/ml) for 30 min, or pretreated with cyclosporin A (5 μM) for 1 h followed by 30-min treatment with IGF-I (50 ng/ml). Cells were fixed in methanol, and subcellular localization of NFATc1 was determined by immunostaining with anti-NFATc1 antibody followed by Texas red conjugated anti-rabbit secondary antibody.

DISCUSSION

In this study, we provide the first evidence that IGF-I plays a direct role in the initiation of hypertrophy of mesangial cells. In addition, we show that IGF-I-mediated hypertrophy is associated with increased expression of the extracellular matrix proteins fibronectin and collagen type IV. Simultaneous exposure of the cells to IGF-I and TGFβ resulted in an additive effect on both hypertrophy and the increase of ECM accumulation, suggesting that these growth factors may act through parallel pathways. IGF-I is a well-characterized mitogen, and it has been speculated that the role of IGF-I in renal hypertrophy is due to its effect on cell proliferation. However, in our experiments, no change in the cell cycle was seen and no significant change in DNA synthesis was observed at the same dose of IGF-I required to induce maximal hypertrophy. At higher doses of IGF-I, there was an increase in DNA synthesis, consistent with other reports in the literature (29, 30).

IGF-I has been shown to signal via Erk1/Erk2 MAPK and PI3K in many different cell types (11). However, the precise

role of each signaling pathway in mediating the biological effects of IGF-I remains to be characterized. Although Erk1/Erk2 MAPK was activated by IGF-I in our studies, it does not appear to be required for induction of hypertrophy. It is still possible that the MAPK pathway might be important for other cellular responses to IGF-I, including migration, modulation of DNA synthesis, and cell survival. Treatment of MCs with IGF-I also resulted in the activation of PI3K and Akt kinase. Interestingly, the serine/threonine kinase Akt is activated by IGF-I in a bi-phasic manner. IGF-I induced rapid activation of Akt (10 min) that was inhibited by wortmannin, demonstrating that this activation is mediated by PI3K. However, wortmannin had no effect on the delayed phase of Akt activation, indicating a PI3K-independent mechanism of activation. Inhibition of PI3K by wortmannin or LY294002 had no effect on IGF-I-induced hypertrophy, demonstrating that PI3K is not required for this biological effect of IGF-I. However, the sustained activation of Akt *was* inhibited by cyclosporin A, indicating that activation of calcineurin by IGF-I is responsible for the sustained Akt activation in MCs. Our data support other reports in the literature that calcineurin activation can act in concert with other signaling pathways (31, 32). The consequences of sustained Akt activation by calcineurin and the role of Akt in IGF-I-mediated hypertrophy remain to be evaluated.

Our data show that IGF-I treatment of MCs not only increases the levels of calcineurin protein, but also calmodulin. As calcineurin activation is dependent upon availability of calmodulin (33), one possible consequence of increased calmodulin is sustained activation of other calcium/calmodulin pathways and/or priming of the calcineurin/calmodulin system for activation by other signals. Interestingly, the increase in both calcineurin and calmodulin occurs rapidly following IGF-I treatment, particularly in comparison to the global increase in protein reflected in the protein/DNA ratio. This suggests that the IGF-I-mediated increase in calcineurin and calmodulin protein expression is a specific action of IGF-I and not merely a consequence of the generalized increase in protein synthesis associated with hypertrophy. However, a more dynamic assay of protein synthesis such as incorporation of [^{35}S]methionine into proteins would more accurately address the contribution of global protein synthesis to the increased expression of calcineurin and calmodulin. Moreover, our data do not address the contribution of protein degradation to the modulation of calcineurin/calmodulin levels.

Interestingly, calcineurin activation occurred fairly late after IGF-I treatment. Both Erk1/Erk2 MAPK and Akt are phosphorylated within minutes of IGF-I addition and peak activation has subsided in less than 1 h. Calcineurin activity appears to peak ~2 h following addition of IGF-I and returns to baseline by 4–6 h. Additionally, calcineurin activation precedes a significant increase in calcineurin and calmodulin protein levels, as well as induction of hypertrophy by IGF-I. Our data show that this increase in phosphatase activity is in fact required for IGF-I-mediated hypertrophy. Inhibition of calcineurin with cyclosporin A completely blocks IGF-I-mediated activation of calcineurin phosphatase activity and IGF-I-mediated hypertrophy and ECM accumulation. These effects of cyclosporin A on matrix protein accumulation were specific, because the compound had no effect on total protein synthesis or on the levels of other proteins such as Erk1/Erk2. Of interest is the finding in Fig. 6*A* that increased intracellular calcium due to treatment with ionophore A23187 did not induce hypertrophy. This is consistent with the observation, reported by Musaro *et al.* (34), that calcium alone is actually toxic to some cells. Instead, there appears to be a requirement for an additional IGF-I-mediated signal to generate a hypertrophic response. One possibility is cross-talk with other signaling pathways such as Akt (35, 36).

Several proteins have been identified as targets of calcineurin-mediated dephosphorylation, including NFAT, MEF2A and -2D, and GATA (15, 33). Our data demonstrate that calcineurin is important in IGF-I-mediated hypertrophy. Therefore, calcineurin substrates may also be critical signaling molecules that mediate hypertrophy in MCs. IGF-I induces nuclear localization of NFATc1, which is dependent upon calcineurin activity. Therefore, NFATc1 may be important in transcriptional regulation of hypertrophic response genes, including ECM genes and possibly even of calcineurin and calmodulin. It remains to be seen whether other calcineurin substrates also play a role in the regulation of genes responsible for cell hypertrophy.

The demonstration that IGF-I activates a calcium-dependent serine/threonine phosphatase in mesangial cells introduces a new paradigm in IGF signaling. In addition to its role as a mitogen, requiring primarily the MAPK and PI3K signaling pathways, our work and the work of other groups in this area show that IGF-I elicits relevant biological responses via calcium-dependent signaling pathways, including, but possibly not limited to, calcineurin phosphatase. Similarly, this study demonstrates another potential target tissue for the action of calcineurin. Discovering how growth factors such as IGF-I activate calcineurin and what factors downstream of calcineurin are required for inducing hypertrophy will be important areas for further research. Furthermore, calcineurin represents an intriguing new pathway with substantial relevance in renal disease. Treatment of animal models of cardiac hypertrophy with inhibitors of calcineurin have been successful at preventing hypertrophy (22, 26, 32); therefore, it is possible that calcineurin inhibitors may also be of therapeutic value in renal diseases characterized by hypertrophy.

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REFERENCES

1. Wolf, G., and Ziyadeh, F. N. (1999) *Kidney Int.* **56,** 393–405
2. Gronbaek, H., Volmers, P., Bjorn, S., Osterby, R., Orskov, H., and Flyvbjerg, A. (1997) *Am. J. Physiol.* **272,** E918–E924
3. Verotti, A., Cieri, F., Petitti, M., Morgese, G., and Chiarelli, F. (1999) *Diabetes Nutr. Metab.* **12,** 271–276
4. Flyvbjerg, A. (1997) *Kidney Int.* **52,** S12–S19
5. Feld, S., and Hirschberg, R. (1996) *Endocr. Rev.* **17,** 423–480
6. Sugimoto, H., Shikata, K., Makino, H., Ota, K., and Ota, Z. (1996) *Nephron* **72,** 648–653
7. Doi, T., Striker, L. J., Quaife, C., Conti, F. G., Palmiter, R., Behringer, R., Brinster, R., and Striker, G. E. (1988) *Am. J. Pathol.* **131,** 398–403
8. Quaife, C. J., Mathews, L. S., Pinkert, C. A., Hammer, R. E., Brinster, R. L., and Palmiter, R. D. (1989) *Endocrinology* **124,** 40–48
9. Flyvbjerg, A., Marshall, S. M., Frystyk, J., Hansen, K. W., Harris, A. G., and Orskov, H. (1992) *Kidney Int.* **41,** 805–812
10. Flyvbjerg, A., Bennet, W. F., Rasch, R., Kopchick, J. J., and Scarlett, J. A. (1999) *Diabetes* **48,** 377–382
11. Yenush, L., and White, M. (1997) *Bioessays* **19,** 491–500
12. di Mari, J., Davis, R., and Safirstein, R. (1999) *Am. J. Physiol.* **2777,** F195–F203
13. Horney, M. J., Shirley, D. W., Kurtz, D. T., and Rosenzweig, S. A. (1998) *Am. J. Physiol.* **274,** F1045–F1053
14. Shultz, P., DeCorleto, P., Silver, B., and Abboud, H. (1988) *Am. J. Physiol.* **255,** F674–F684
15. Blaser, F., Ho, N., Prywes, R., and Chatila, T. (2000) *J. Biol. Chem.* **275,** 197–209
16. Choudhury, G. G., Karamitsos, C., Hernandez, J., Gentilini, A., Bardgette, J., and Abboud, H. E. (1997) *Am. J. Physiol.* **273,** F931–F938
17. Fruman, D. A., Pai, S.-Y., Klee, C. N., Burakoff, S. J., and Bierer, B. E. (1996) *Methods Enzymol.* **9,** 146–154
18. Abboud, H. E. (1997) *Kidney Int.* **52,** S3–S6
19. Choi, M., Eung-Gook, K., and Ballerman, B. (1993) *Kidney Int.* **44,** 948–958
20. Schreiber, B., Hughes, M., and Groggel, G. (1995) *Clin. Nephrol.* **43,** 368–374
21. Pricci, F., Pugliese, G., Romano, G., Romeo, G., Locuratolo, N., Pugliese, F., Mene, P., Galli, G., Casini, A., Rotella, C., and Di Mario, U. (1996) *Endocrinology* **137,** 879–885
22. Sussman, M. A., Lim, H. W., Gude, N., Taigen, T., Olsen, E. N., Wieczorek, D. F., and Molkentin, J. D. (1998) *Science* **281,** 1690–1693
23. Molkentin, J., Lu, J., Antos, C., Markham, B., Richardson, J., Robbins, J., Grant, S., and Olsen, E. (1998) *Cell* **93,** 215–228
24. Schreiber, S., and Crabtree, G. (1992) *Immunol. Today* **13,** 136–142
25. Tumlin, J. A. (1997) *Am. J. Kidney Dis.* **30,** 884–895
26. Lim, H. W., De Windt, L. J., Steinberg, L., Taigen, T., Witt, S. A., Kimball,

T. R., and Molkentin, J. D. (1999) *Circulation* 2431–2437
27. Chen, L., Glover, J., Hogan, P., Roa, A., and Harrison, S. (1998) *Nature* **392,** 42–48
28. Rao, A., Luo, C., and Hogan, P. (1997) *Annu. Rev. Immunol.* **15,** 707–747
29. Grellier, P., Sabbah, M., Fouqueray, B., Woodruff, K., Yee, D., Abboud, H. E., and Abboud, S. L. (1996) *Kidney Int.* **49,** 1071–1078
30. Fled, S. A., Hirschberg, R., Artishevsky, A., Nast, C., and Adler, S. G. (1995) *Kidney Int.* **48,** 45–51
31. Molkentin, J. (2000) *Circ. Res.* 731–738
32. Murat, A., Pellieux, C., Brunner, H.-R., and Pedrazzini, T. (2000) *J. Biol. Chem.* **275,** 40867–40873
33. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) *Nat. Rev.* **1,** 11–23
34. Musaro, A., McCullagh, K. J., Naya, F. J., Olsen, E. N., and Rosenthal, N. (1999) *Nature* **400,** 581–585
35. Yoshimoto, T., Uchino, H., He, Q. P., Li, P. A., and Siesjo, B. K. (2001) *Brain Res.* **899,** 148–158
36. De Windt, J., Lim, H., Taigen, T., Wencker, D., Condorelli, G., Dorn, G. N., Kitsis, R., and Molkentin, J. (2000) *Circ. Res.* **86,** 255–263