AD NUMBER

ADB282106

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Oct 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 21 Feb 2003

AD_____

Award Number: DAMD17-99-1-9021

TITLE: Tyrosine Kinase Display of Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Hsing-Jien Kung, Ph.D.

CONTRACTING ORGANIZATION: University of California, Davis
Davis, California 95616

REPORT DATE: October 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-99-1-9021
Organization: University of California, Davis

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Nm Sinsha anan Kum
06/19/02

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Final (1 Apr 99 - 30 Sep 01)

4. TITLE AND SUBTITLE
Tyrosine Kinase Display of Prostate Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9021

6. AUTHOR(S)
Hsing-Jien Kung, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Davis
Davis, California 95616

E-mail: hkung@ucdavis.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Oct 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

In this proposal, we present an innovative approach, tyrosine kinase display, to rapidly identify tyrosine kinases differentially expressed in prostate carcinomas. Our hypothesis is that tyrosine kinases play central roles to the growth and progression of prostate carcinomas. Knowledge of tissue- or stage-specific tyrosine kinases could potentially provide better prognostic markers and intervention strategies. The innovative PCR-based approach developed here requires only restriction digestion and gel electrophoresis, and allows all or nearly all expressed tyrosine kinases to be directly "read" from the gel. It is rapid, quantitative, and insensitive to RNA degradation and requires minimal amounts of RNA. When fully developed, it is expected to have wide-applications in both basic and clinical settings. In the past two and half years (May, 1999 to Sept. 2001), we have made significant progress, notably 1). We have established comprehensive tyrosine kinase profiles for all six widely used prostate cancer cell lines and three LNCaP androgen-independent variant cell lines, as well as those of immortalized prostate epithelial cells and the normal primary cultures of prostate stromal and epithelial cells; 2). We have identified several kinases differentially expressed in prostate cancer cells, in androgen-dependent as well as -independent cells. These are potential prognostic markers and intervention targets; 3). We have identified a new kinase whose expression is induced by DHT and which may mediate signals channelled by androgen receptor. Modulation of this kinase activity may restore androgen sensitivity or block androgen independent growth; and 4). We have validated the important biological roles of the Etk, the first novel tyrosine kinase uncovered by tyrosine kinase profile, in prostate cancer. Thus, this proposal has taken us from the initial profiling of tyrosine kinases to the identification of potential markers for prostate cancers, and to the demonstration of functions of these tyrosine kinases.

14. SUBJECT TERMS

15. NUMBER OF PAGES
56

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

Tyrosine kinases, while a minor class of protein kinases, represent a major class of oncogenes. They are involved in the growth and metastasis of prostate cancer cells (for examples, 1-3) and play key roles in tumor sensitivity to radiation and chemical-induced apoptosis. They are valuable prognostic markers and important targets for intervention (2,4). Kinase inhibitors have recently shown tremendous efficacies and promises in the treatment of human cancers (for reviews see 7 and 8; 9,10). Thus, there is a need to identify the tyrosine kinases expressed in a cancer cell, especially the differentially expressed ones. It has been estimated that there are about 1000 to 2000 protein kinases encoded by the human genome and about 100 of them (i.e., 10%) are tyrosine kinases (6). With nearly all the human genome sequences determined, we now have an accurate count of the number of tyrosine kinases encoded by human genome. In a recent *Oncogene* review written by my graduate students and postdoctors (24), we showed that there are human 90 tyrosine kinase genes and 6 pseudogenes. In a given cell at a given stage, 30 to 50 of them are expressed, a number that is large enough to give tissue or tumor specific characteristics, but small enough to be determined by a simple screen. The present proposal describes an innovative and effective means to display expressed tyrosine kinases of a given prostate cancer cell type, using a single RT-PCR reaction and analyzed by a single gel. Aberrantly expressed or novel tyrosine kinases can be readily identified. There are two major tasks of this proposal:

1. To develop a complete tyrosine kinase display of prostate carcinomas.
2. To identify tyrosine kinases expressed in responses to hormones, drugs and extracellular stimuli.

BODY OF PROGRESS REPORT (April, 1999 to Oct. 2001)

Task 1: To develop a comprehensive tyrosine kinase display of prostate cancer cells.

Comprehensive tyrosine kinase profiles of prostate cancer cells

We have made significant progress toward this aim. A comprehensive, if not exhaustive, analysis of the tyrosine kinase content of 5 prostate cancer cell lines, LNCaP, CWR22R, DU145, PC3 and MDA-Pca2b, TsuPr1 has been done. MLCSV40 was used as a control for normal prostate epithelial cells, so were PrSC (prostate stromal cells) and PrEC (prostate epithelial cells), normal primary cultures purchase from Clonetics. In addition, we developed the tyrosine kinase profiles of three androgen-independent LNCaP variant cell lines (cds1 to 3), derived by our colleague Dr. Xubao Shi. These profiles provide enormous amount of information about signal pathways, as tyrosine kinases are key transmitters of signal transduction. We found that each cell, cancer or normal, generally expresses about 30 to 40 tyrosine kinases, many of which have potential to interact or crosstalk with each other to form a signal network. It expresses a total of 34 tyrosine kinases, of which 21 are receptors and 13, non-receptors. The receptor tyrosine kinases include erbB1, erbB2, erbB3, ephA1, ephB1, ephB2, ephB3, ephB4, Nyk, Sky, Ddr1, Ddr2, PDGFRb, FGFR1, FGFR2, IGFR, MET, Ron, Ret, TrkA, and VEGFR. The non-receptor tyrosine kinases are src, fyn, yes, brk, frk, csk, etk, abl, arg, jak1, tyk2, fak, and pyk2. We also conducted a microarray analysis (UA95, Affymetrix) of the same sample, and found that the tyrosine kinase display approach described here is much more sensitive for low-copy number tyrosine kinases.

In general, the androgen responsive prostate cell lines display a pattern more similar to each other than to androgen-independent cell lines. For instance, FGFR4 is expressed in androgen-independent DU145 and PC3, but not in LNCaP and CWR22R. Likewise, ErbB3 is highly expressed in androgen dependent lines LNCaP and CWR22, but much less in DU145 and PC3. By contrast, Axl kinase is suppressed in LNCaP and CWR22, but not in DU145 and PC3. These data, taken together raise the possibility that some of the kinases such as Axl, RON and FGFR4 may be involved in androgen independence, whereas ErbB3 may be involved in differentiation phenotype (as both DU145 and PC3 are more undifferentiated). This is consistent with the our previous results that treatment of LNCaP by neuregulin or heregulin , the ligand for erbB3 induces cell spreading, stress-fiber formation and the acquisition of a more epithelial-differentiation phenotype (19). In collaboration with Ming-Chie Hung's lab, the tyrosine kinase display approach was used to study kinases that are involved in breast cancer progression. Interestingly, a kinase that is expressed in cancer cells resistant to apoptosis, but suppressed in E1A transfected cells, which undergoes apoptosis turns out to be Axl (11). These results taken together suggest that Axl may be involved in the protection of hormone-sensitive cancer cells from hormone-withdraw induced death. Without Axl, the cancer cells (LNCaP, CWR22 and E1A treated breast cancer cells) remain highly sensitive to hormone. Conversely, overexpression of Axl may be one reason why these cells become hormone independent. Further pursuit of the relationship of Axl and hormone independence seems to be warranted. In addition, perhaps non-coincidentally, Mer/Nyk, the kinase found overexpressed in prostate cancer but not normal cells is a relative of Axl. Based on a comparison of the tyrosine kinase profiles of normal prostate cells, PrSC and PrEC with all other cancer cell lines reveal that Nyk/Mer is consistently expressed at a higher level. Nyk/mer is a kinase originally cloned by us (16, 18) and independently Dr. Earp's lab at UNC, Chapel Hill (17). We showed previously that Nyk has a high transforming potential (16), consistent with its being involved in prostate cancer progression. We also found that the third member of this family of receptor tyrosine kinase, Sky, is also overexpressed in prostate cancer cells. These kinases as a family are generally involved in cell adhesion and cell movement. DU145 and PC3, in which Axl and Sky are highly expressed are known to be involved in cell motility and invasion. The involvement of this family of kinases in metastasis of prostate cancer deserves future attention.

* the complete tyrosine kinase profiles of prostate cancer cells are being included in a manuscript under preparation to be submitted to Nature Medicine

Validation and characterization of the tyrosine kinases uncovered by TK profile

As described in the previous progress report, our task in the last year of the grant period was to validate the expression of the tyrosine kinases uncovered in the tyrosine kinase profiles of prostate cancers. As described above, the Axl family kinases will be a focus and the other one is Etk, which was originally cloned from CWR22 cells and shown to be critically involved in prostate biology. Due to the limited time remained in the grant, we chose to focus on Etk.

The first novel kinase uncovered based on tyrosine kinase profiles is Etk (25,26). Because of its relevance to prostate growth and biology, we made a conscious effort to study this kinase and set up a number of productive collaborations to fully characterize this kinase. Etk is a new member of the Btk family of kinases (27), which distinguish themselves from others by having a pleckstrin-

homology (PH) domain at the N-terminus (27,28,29,30). Btk was uncovered as a kinase whose germ-line mutation leads to defects in B-cell development or Bruton's syndrome. Soon after, ITK and Tec, the other members of this family were discovered and found to be critical in T cell development. Etk is the newest member of this family. Unlike, Btk, Itk and Tec which are primarily associated with hematopoietic cells, Etk is predominantly expressed in epithelial and endothelial cells, (and hence the name Etk).

Our progress on Etk is summarized as follows. We showed that Etk plays an important role in antiapoptosis, motility and neuroendocrine differentiation of CaP cells (25,31). It transforms epithelial cells when overexpressed and its expression is elevated in metastatic cancer cells (32). The reason that Etk can engage several signal pathways is likely due to its modular structure allowing it to form complex with a large number of signal molecules. In addition to the PH domain, Etk contains a SH3 domain and a SH2 domain, all involved in protein-protein interaction (27). The PH domain also binds lipid, PI (3,4,5) P3. In the unbound form, the PH domain negatively regulates Etk activity. Removal of the PH domain by caspase cleavage (33) or its binding to proteins such as PTPD1 (34) and FAK (31) all result in "opening" up the kinase domain. This allows Src-like kinases to phosphorylate tyrosine residue 566, leading to maximal activity (32,35). The attached figure illustrates how FAK can activate Etk by serving as an anchor site for both Etk and Src.

We know very little about the downstream effectors of Etk. Our work and our collaborators' work have identified STAT1, 3, and 5 as direct substrate of Etk, and can account for the activation of cyclin D and p27 by Etk. In addition, PAK, p21-activated kinase, is also a direct substrate of Etk. PAK is known to activate JNK and p38 MAPK pathways (36). By analogy to other members of the Btk family members, likely there are more effectors to be discovered in the future.

As one of the objectives to validate the functional significance of discovered new tyrosine kinases, we asked the question whether Etk is involved in prostate cancer progression, especially at the stage of transition from androgen dependence to independence. Based on the tyrosine kinase profiles, we know that src, FAK and Etk are all expressed in LNCaP, an androgen-dependent prostate cancer cell line. From our work as well as others, we know that Fak associates with both Src and Etk but at different sites. There has been considerable interest in understanding whether neuroendocrine differentiation of prostate epithelial cells plays an important role in prostate cancer progression. The number of neuroendocrine cells increases in advanced prostate cancers and serum chromograinin and neuropeptide level is higher in androgen-independent prostate cancers. The latter molecules are markers of neuroendocrine differentiation. Gastrin-releasing peptide (homolog of amphibian bombesin) and neurotensin are neuropeptides released by neuroendocrine cells after trans-differentiated from prostate epithelial cells. Bombesin is known to activate src and Fak, through G-protein coupled receptors. We therefore investigated whether Etk, or the Src-Fak-Etk complex, is involved in the transition between androgen-dependence to independence. Our hypothesis is that neuropeptides such as bombesin and neurotensin can under certain circumstances substitute for androgen, and their signals through tyrosine kinases activate androgen receptor (in the absence of ligand). Our study in the last year provided strong evidence for this hypothesis. Taking advantage of our precise knowledge of the variety of tyrosine kinases in LNCaP, we studied the activation of the 10 non-receptor tyrosine kinases (the antibodies of the remaining three are not of high quality and the results were inconclusive) upon bomebsin treatment, and found indeed Src, Fak and Etk are prominently activated. We then showed that bombesin can substitute androgen to sustain the growth

of LNCaP in charcoal-stripped media and thus can serve as a progression factor, converting LNCaP from androgen-dependent to independent state. This conversion requires Etk, FAK and Src, as dominant-negative mutants of these kinases or selective inhibitors of these kinases block this conversion. Our results uncover one mechanism whereby androgen-independence can be achieved and suggest that tyrosine kinase inhibitors, which have shown significant promises in cancer treatments, may be used in conjunction with anti-hormone in the treatment of prostate cancer. Importantly, these results illustrate the utility of tyrosine kinase profiling and verify the expression analysis.

*The results will be published in Mol. Cell Biol. 2001, in press.

Other results Using Cy3 and Cy5-dCTP (Amersham) during the reverse-transcription-polymerase-chain reaction, we have done a systematic analysis of using capillary electrophoresis to study the digestion profile, in an effort to automate the display. The results are clear and promising. However, we also found that the quantitation is not as good as phosphoimaging of the autoradiogram. The intensity range using fluorescence is relatively small. Thus this approach will allow us to identify the types of kinases in a sample with high throughput set up, but the quantitation or the expression level measurement will be compromised. We have also surveyed 5 tumor specimens with matched normal control samples. We face the same problems as others dealing with microarray analysis; the heterogeneity of the samples makes the interpretation difficult. We are anxiously waiting to improve the signal amplification approach so that laser-dissected samples can be used. Thus far, the results have not been promising.

Task 2: To Identify tyrosine kinases expressed in responses to hormones, drugs and extracellular stimuli.

Androgen treatment With androgen treatment, we have identified a novel kinase AIK (androgen induced kinase), the human homolog of rat MAK (male associated kinase) that is activated about 6 fold at the transcriptional level (20, 23). A full-length cDNA was cloned and used to study its expression level. The expression of this kinase is restricted to testis and hence the term, male-associated kinase. Normal prostate tissues do not express this kinase at a high level nor do the cancer tissues. However, after androgen treatment, the expression level of this kinase becomes higher. To confirm this observation, a real-time PCR was used to quantify the level of MAK in LNCaP cell line after androgen treatment. Real time PCR is a recently developed approach, which permits precise quantitation of the RNA concentration by monitoring the reaction kinetics of the PCR, thus making PCR quantitative (21). Our lab has a Biorad, iCycler instrument, which allows us to conduct such an analysis. Briefly, specific primers that can amplify MAK transcripts are used to generate RT-PCR products. The thermocycle at which the reaction reaches 50% yields the rate of reaction, which is proportional to the initial concentration of the transcript. Different concentrations of DHT (synthetic androgen) were used and the results are summarized in Fig. 3. The induction is at the highest level when 10 nm DHT is used; higher or lower concentrations all have less potency. There are two conclusions drawn from this result. First, it is clear from this quantitative analysis that MAK expression is induced by DHT, confirming the tyrosine kinase display results, and attests to the sensitivity of the display approach (the expression level of MAK is so very low, beyond the detection by standard Northern analysis). Second, the dose response curve of MAK induction

parallels that of the growth response, indicating that MAK may be involved in mediating DHT induced growth of LNCaP cells. To our knowledge this is the first protein kinase, which is induced by androgen at the transcription level. Together with the recent finding that androgen signals induces MAP kinase (22), this result strengthens the hypothesis that androgen receptor signaling may involve kinase and phosphorylation cascade. It will be of great interest to study the kinases involved in such as cascade. It is conceivable that deregulation of some of these downstream kinases may account for androgen-independent growth. Inhibition for these kinases could either terminate the constitutive signals or restores the hormone-sensitivity. Thus, there is a great need to understand the nature and the mechanisms of action of MAK. From the structural data, we know this kinase is in the dual-kinase family (i.e., sequences are similar to kinases which phosphorylate both serine/threonine and tyrosine residues). The catalytic domain carries motifs analogous to both MAPK and cdc2. We also know now that there are two other related kinases in the same family. They are MRK and MOK. MOK is also testis specific, but does not seem to be induced transcriptionally by androgen; MRK is ubiquitously present in many tissues.

Growth induction: In the original proposal, we also wished to test whether growth conditions would alter the tyrosine kinase expression profiles. We did a careful analysis of the cell density effect on the expression profiles. There are two reasons to conduct this type of experiment; first, when the cells reach certain density, they usually slow down their growth and the tyrosine kinase profile may identify kinases involved in this process. Second, the increased density facilitates cell-cell communication and we may be able to identify kinases that respond to cell-adhesion signals. Tsu-Pr1 cell line was used to test how cell-cell communication may affect the kinase expression. Cells grew to 30%, 70%, 100% or >100% (i.e., piling up) confluency were used. An example is given on the right panel of Fig. 1., where RNA isolated from Tsu-Pr1 cells grown to different density was subject to RT-PCR and restriction digestion. The kinases and their expression level were identified as before. We found that the expression of most of the kinases was not affected by cell density. There are however, a few interesting exceptions. The expressions of EGFR and FGFR3 decrease, as cell density increase, whereas those of RON and JAK3 increase. It would be of interest to define the nature of the cell-cell communication that mediates this transcriptional regulation and to understand the roles of the above kinases in regulating growth and senescence. This project is ongoing and will be extended to the next grant period.

Validation and characterization of kinases induced by ligands

As in task1, we have focused on defining the functional significance of the uncovered kinases based on TK profiling in the last year of the grant period. We wish to demonstrate that increased expression of MAK by androgen treatment as detected by TK profiling is not an artifact or a peculiarity of LNCaP cells. To this end, To this end, we isolated a 4 kb 5'flanking segment containing the promoter of MAK gene from a BAC library of human genome. A luciferase reporter gene is linked to the promoter and transfected into LNCaP cells, followed by treatment with DHT at different concentrations and at different time points. The results completely confirm the tyrosine kinase profile result in that the MAK promoter is activated about 6 fold at 1 nM of DHT. The kinetics of induction and the dose-response curves coincide with those obtained by real time RT-PCR. These results verify the sensitivity and accuracy of the TK profiling approach and also suggest that the induction by

hormone is primarily at the transcription stage. Within the promoter, there are several ARE (androgen responsive element) and ARR (androgen responsive related element), potential binding sites for androgen receptor. Ongoing experiment investigates whether these elements are responsible for the induction. If so, this is a gene directly targeted by androgen receptor, a transcriptional factor, which can serve as another indicator for androgen receptor activity in prostate cancer cells. We also made significant stride in understanding the possible role of MAK in androgen signaling. Our hypothesis is that this kinase would facilitate the transcriptional activity of androgen receptor by phosphorylating either the receptor itself or coregulators associated with the receptor. We found that MAK directly associated with AR. Whether it phosphorylates AR and other coactivators remain to be established. Again, these are extremely interesting leads that may shed lights into androgen signaling pathways and attests to the power of the tyrosine kinase profile approach.

*A manuscript on the discovery of MAK and its transcriptional activation by DHT is being written up for submission to J. Biol. Chem.

Other results

We have conducted an extensive analysis of possible tyrosine kinases transcriptionally regulated by cytokines, apoptosis inducing agents, growth factors, and hormones. While subtle variations have been observed, in general, most of the tyrosine kinases are not regulated at the transcriptional level, which is consistent with their activation by phoshorylation and posttranslational modifications. As reported above, we did identify at least one, MAK, which is consistently upregulated by DHT and confirmed it is at the transcriptional level by the isolation of the promoter. Our results on density-dependent activation of tyrosine kinases are interesting. Unfortunately, the cell line we used to study the density dependence, Tsu-pr1, turns out to be of bladder origin as recently uncovered by Dr. Gary Miller's lab. While this does not diminish the finding, but it becomes less relevant to this proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Established comprehensive tyrosine kinase profiles for 10 prostate cancer cell lines (LNCaP, CWR22, CWR22R, DU145, PC3, Tsu-Pr1, MDA-PCA2b, cds1, cds2, cds3), one immortalized prostate epithelial cells (MCSV40), and two primary prostate cultures of stromal and epithelial cells.
- Identified several kinases whose expressions vary among androgen-dependent and –independent cell lines.
- Uncover tyrosine kinase Etk and its critical role in androgen-independent growth associated with neuroendocrine differentiation.
- Discovery of a new kinase whose expression is induced by DHT, an androgen analogue.
- Uncover the dual kinase MAK and shown that it's promoter is transcriptionally activated by DHT, and that it associates with androgen receptor and enhances the transcription by androgen receptor.
- The tyrosine kinase display approach developed here has now been used by at least 10 laboratories, which resulted in the identification of at least six tyrosine kinases as potential tumor markers for colon cancer, gastric cancer, breast cancer and EBV-transformed cells.

REPORTABLE OUTCOMES

Hsing-Jien Kung, Clifford G. Tepper, and Ralph W. deVere White. Tyrosine kinases and cellular signaling in prostate cancer (2000) pp241-266 *Prostate Cancer: Biology, Genetics and the New Therapeutics* ed. by Chung L.W.K, Isaacs W.B., and Simons J. W.. Human Press Inc., Totowa, NJ

Yun Qiu and Hsing-Jien Kung. Signaling network of the Btk family kinases (2000) Oncogene 19:5651-5661.

Li-Fen Lee, Junlin Guan, Yun Qiu and Hsing-Jien Kung. Neuropeptide-induced androgen independence in prostate cancer cells: the roles of non-receptor tyrosine kinases Etk/Bmxc, Src and FAK. (2001) Molecular Cell Biology, 21(24) Dec.

CONCLUSIONS

The conclusion of this proposal has led to the identification of an androgen inducible kinase MAK, which enhances the transcriptional activity of androgen receptor and is associated with androgen receptor. This kinase promises to play a key role in androgen signalings and may be involved in prostate cancer progression. The results of this proposal also uncovered a new tyrosine kinase Etk, involved in neuroendocrine differentiation and neuropeptide-induced androgen independence. This kinase forms a complex with src and FAK, and is critically involved in antiapoptosis and cell motility. In addition, the tyrosine kinase profile approach has led to the discovery of at least one member of the Axl family of kinases (e.g., Axl, Nyk or Sky) is overexpressed in prostate cancers. These kinases are known to be involved in antiapoptosis and cell migration. They are potential targets for inhibition. The tyrosine kinase display approach developed by this proposal offers an efficient and rapid approach to identify the content and quantities of tyrosine kinases. Since tyrosine kinases are master switches controlling a variety of cellular responses including growth, apoptosis, differentiation, migration, metastasis, chemo- and radio-sensitivity, knowledge about the kinases involved opens avenues to 1). Understand the complex signal pathways; 2). Design strategies for modulating the behavior of cancer cells; 3) Sensitize cancer cells toward chemo- and radiotherapies; 4) Develop inhibitors for kinases or associated signal molecules to inhibit cancer cell growth. 5) Develop agents that may restore hormone-dependence or eliminate hormone-independent cells. As described, the protocol of our approaches have already been shared with al least 10 labs, resulting at least 8 publications by various labs.

REFERENCES:

1. Ware, J.L. (1993) Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. Cancer Metastasis Rev. 12:287-301.

2. Zhau, H.E., Pisters, L.L., Hall, M.C., Zhao, L.S., Troncoso, P., Pollack A., Chung, L.W. (1994) Biomarkers associated with prostate cancer progression. J. Cell Biochem Suppl. 19:208-216.

3. Humphrey, P.A., Zhu, X., Zarnegan, R., Swanson, P.E., Ratliff, T.L., Vollmer, R.T., Day, M.L. (1995) HGF and its receptor c-met in prostatic carcinoma. Am. J. Pathol. 147:386-396.

4. Levitzki, A. and Gazit, A. (1995) Tyrosine kinase inhibition: an approach to drug development. Science 267:1782-1792.

5. Robinson, D., He, F., Pretlow, T.G., Kung, H.J. (1996) A tyrosine kinase profile of prostate carcinoma. Proc. Natl. Acad. Sci. USA, 93:5958-5962.

6. Hanks, S., and Hunter, T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J., 9: 576-596.

7. Shawver, L.K. (1999) Tyrosine kinase inhibitors:from the emergence of targets to their clinical development. Clinical Oncology,. 29-47

8. Woodburn, J.R. (1999) The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol. Ther. 82: 241-250

9. Huang, S.M., Bock, J.M., and Harari, P.M. (1999) Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis and radiosensitivity in squamous cell carcinomas of the head and neck. Cancer Res. 59: 1935-1940.

10. Fong, T.A.T., R., Shawver, L.K., Sun, L., Tang, C., App, H., Powell, J.T., Kim , Y.H., R., Wang, X., Risau, W., Ullrich, A., Hirth, K. P., and McMahon, G. (1999) SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res. 59: 99-106.

11. Lee, W.P., Liao, Y., Robinson, D., Kung, H.J, Liu, E. T. and Hung, M.C. (1999) Axl-gas6 interaction counteracts E1A-mediated cell growth suppression and propapoptotic activity. Mol Cell Biol. 19: 8075-8082.

12. Wainstein, M.A., He, F., Robinson, D., Kung, H.J., Schwartz, S., Giaconia, J.M., Edgehouse, N.L., Pretlow, T.P., Bodner, D.R., Jursh, E.D., Resnick, M.I., Seftel, A., and Pretlow, T.G. (1994) CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Research, 54:6049-6052.

13. Naganbushan, M., Miller, C.M., Pretlow, T.P., Giaconia, J.M., Edgehouse, N.L., Schwartz, S., Kung, H.J., de Vere White, R.W., Gumelock, P.H., Resnick, M.I., Amini, S., and Pretlow, T.G. (1996) CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Research, in press.

14. Pretlow, T.G., Wolman, S.R., Micale, M.A., Pelley, R.J., Kursh, E.D., Resnick, M.I., Bodner, D.R., Jacobberger, J.W., Delmoro, C.M., Giaconia, J.M., Pretlow, T.P. (1993) Xenografts of primary human prostatic carcinoma. J. Natl. Cancer Inst. 85:394-398.

15. Cheng, L., Sun, J., Pretlow, T.G., Culp, L.J., Yang, N.S. (1996) CWR22 xenograft as an ex vivo human tumor model for prostate cancer. J. Natl Cancer Inst. 88:607-611.

16. Ling, L., and Kung, H.J. (1995) Mitogenic signals and transforming potential of Nyk, a newly identified N-CAM related receptor tyrosine kinase. Mol. Cell Biol., 15: 6582-6592.

17. Graham, D.K., Dawson, T.L., Mullaney, D.L., Snodgrass, H.R., and Earp, H.S. (1994) Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. Cell Growth Differ. 5:647-657.

18. Ling, L., Templeton, D.,Kung, H.J. (1996) Identification of the major autophosphorylation sites of Nyk/mer, an N-CAM-related receptor tyrosine kinase. J. Biol. Chem., 271 : 18355-18362.

19. Grasso, A.W., Wen, D., Miller, C.M., Rhim, J.S., Pretlow, T.G., and Kung, H.J. (1997) ErbB kinases and NDF signaling in human prostate cancer cells. Oncogene,15 : 2705-2716 .

20. Xia, L., Robinson, D., Chen , H.C., Ma, A.H., and Kung, H.J., (2000) AIK, a novel androgen-inducible kinase, identified by tyrosine kinase display of prostate cancer cells. Proceedings of AACR March 2000, 41: 252

21. Kang, J.J., and Holland, M. J., (1999) PCR applications, protocols for functional genomics, pp429-444. Academic Press, London. Ed. Innis, M.A.

22. Peterziel, H., Mink, S., Schonert, A., Becker, M., Klocker, H. and Cato, A.C. (1999) Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 18: 6322-6329.

23. Matsushime, H., Jinno, A., Takagi, N. and Shibuya, M. (1990) A novel mammalian protein kinase gene (mak) is highly expressed in testicular germ cells at and after meiosis. Mol. Cell. Biol. 10:2261-2268.

24. Robinson, D.R., Wu, Y.M. and Lin, S.F. (2000) The protein tyrosine kinase family of the human genome. Oncogene 19:5548-57.

25. Qui, Y., Ravi, L. and Kung, H.J. (1998) Requirement of ErbB2 for signaling by interleukin-6 in prostate carcinoma cells. Nature 398:83-85.

26. Robinson, D., He, F., Pretlow, T., and Kung, H.J. (1996) A tyrosine kinase profile of prostate carcinoma. Proc. Natl Acad. Sci. USA 93:5958-5962.

27. Qui, Y. and Kung, H.J. (2000) Signaling network of the Btk family kinases. Oncogene 19:5651-5661.

28. Tamagnone, L., Lahtinen, I., Mustonen, T., Virtaneva, K., Francis, F., Muscatelli, F., Alitalo, R., Smith, C.I., Larsson, C., and Alitalo, K. (1994) BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. Oncogene 9:3683-3688.

29. Tsukada, S., Saffran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Mohandas, T., and Quan, S. (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 72:279-290.

30. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F. Hammarstrom, L., Kinnon, D., Levinsky, R., and Bobrow, M. (1993) The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature 361:226-233.

31. Chen, H., Tini, M., and Evans, R.M. (2001) HATs on and beyond chromatin. Curr. Opin. Cell Biol. 13:218-224.

32. Tsai, Y.T., Su, Y.H., Fang, S.S., Huang, T.N., Qiu, Y., Jou, Y.S., Shih, H.M., Kung, H.J., and Chen, R.H. (2000) Etk, and Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. Mol. Cell Biol. 20:2043-2054.

33. Wu, Y.M., Huang, C.L., Kung, H.J., and Huang, C.Y. (2001) Proteolytic activation of etk/bmx tyrosine kinase by caspases. J. Biol. Chem. 276:17672-17678.

34. Jui, H.Y., Tseng, R.J., Wen, X., Fang, H.I., Huang, L.M., Chen, K.Y., Kung, H.J., Ann, D.K., and Shih, H.M. (2000) Protein-tyrosine phosphatase D1, a potential regulator and effector for Tec family kinases. J. Biol. Chem. 275:42214-42232.

35. Rawlings, D.J., Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M., Fluckiger, A.C., Witte, O.N., and Kinet, J.P. (1996) Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science 271:822-825.

36. Bagheri-Yarmand, R., Mandal, M., Taludker, A.H., Wang, R.A., Vadlamudi, R.K., Kung, H.J., and Kumar, R. (2001) Etk/Bmx tyrosine kinase activates PAK-1 and regulates the tumorigenicity of breast cancer cells. J. Biol. Chem. 29:29

ACRONYMS:

ARE	Androgen-responsive element
ARR	Androgen-related responsive element
Axl	An oncogene derived from leukemia
BAC	Bacterial artificial chromosome
BTK	Bruton's syndrome tyrosine kinase
CDC2	#2 gene required for cell division cycle
cds1	Cell line derived from Charcoal-deprived serum
CWR-22R	Case Western Reserve #22 relapsed cell line
CY3	A fluorophore #3
CY5	A fluorophore #5
DHT	Dihydroxytestosterone
DU145	Duke University #145 cell line
E1A	Adenovirus early 1A gene
EGFR	Epidermal growth factor receptor
ErbB3	Erythroblastosis type B #3 oncogene
Etk	Epithelial and endothelial tyrosine kinase
Fak	Focal adhesion kinase
FGFR3	Fibroblast growth factor receptor #3
FGRF4	Fibroblast growth factor receptor #4
Itk	Interleukin-induced tyrosine kinase
JAK3	Janus kinase #3 or just another kinase #3
JNK	Jun N-terminal kinase
kb	Kilo base
LNCaP	Lymph node carcinoma of Prostate cancer cell line
MAK	Male-associated kinase
MDA-PCA2b	MD Anderson prostate cancer cell line #2B
Mer/Nyk	A tyrosine kinase gene with the name of Monocyte Erythrocyte and Reproductive tissues/N-CAM related tyrosine kinase
MLCSV40	A normal prostate epithelial cell line immortalized by SV40 genome
MOK	MRK-related kinase
MRK	MAK-related kinase
NM	Nanomolar
P38MAPK	p38- mitogene activated protein kinase
PAK	p21-activated kinase
PC3	Prostate Cancer #3 cell line
PCR	Preliminary chain reaction
PH	Pleckstrin-homology domain
PI(3,4,5)P3	Phosphatidylinositol triphosphates
PrEC	Normal prostate epithelial cells
PrSC	Normal prostate stroma cells
PTPD1	Protein tyrosine phosphotase #D1
RNA	Ribonucleic Acid
Ron	A proto-oncogene
RT-PCR	Reverse transcription-polymerase chain reaction
SH2	src homology #2 domain
SH3	src homology domain
Sky	A tyrosine kinase in the family of Axl
STAT1	Signal transducer and activator of transcription #1
Tec	A tyrosine kinase in the BTK family
TK	Tyrosine Kinase
TsuPr1	Prostate cell line #1, derived from Tsu tumor

LIST OF PERSONNEL:

Hsing-Jien Kung, Ph.D., Principal Investigator

A.H. Chang, Ph.D., Visiting Post-Doc

Li-Fen Lee, Ph.D., Visiting Post-Doc

Yi-Mi Wu, Ph.D., Visiting Post-Doc

APPENDICES:

- Appendix I: Xia, L., Robinson, D., Chen , H.C., Ma, A.H., and Kung, H.J., (2000) AIK, a novel androgen-inducible kinase, identified by tyrosine kinase display of prostate cancer cells. Proceedings of AACR March 2000, 41: 252

- Appendix II: "Tyrosine kinases and cellular signaling in prostate cancer" by Hsing-Jien Kung, Clifford G. Tepper, and Ralph W. deVere White. Prostate Cancer: Biology, Genetics and the New Therapeutics, ed. by Chung L.W.K. Human Press Inc., (2000)

- Appendix III: Lee LF, Guan JL, Qiu Y and Kung HJ. Neuropeptide-induced androgen independence in prostate cancer cells: the roles of non-receptor tyrosine kinases Etk/Bmx, Src and FAK. *MCB* 21 (24) Dec. (2001).

Jak tyrosine kinases and STAT transcription factors are essential components in signal transduction through hematopoietic cytokine receptors. The Jak2 tyrosine kinase is critical in signaling through e.g. EPO and IFN-γ receptors. Constitutive activation of Jak2 due to a chromosomal translocation has been reported to result in leukemia and cytokine independent growth. Members of the Jak tyrosine kinase family share a conserved tandem kinase domain structure. A tyrosine kinase domain is located in the C-terminus and preceded by a pseudokinase domain. The pseudokinase domain has sequence similarity to kinase domains, but its function is currently unknown. Since appropriate regulation of the Jak2 kinase is essential for normal cellular behavior, we analyzed the roles of the different protein domains of Jak2 in its activation. A series of Jak2 deletion constructs was expressed in 293T cells, where activation of Jak2 is ligand-independent. Deletion of the pseudokinase domain activated Jak2 markedly, whereas deletions in the N-terminal region did not affect the activity of Jak2. Deletion of the pseudokinase domain of Jak2 resulted also in enhanced activation of Stat5. Deletion of the kinase domain resulted in catalytically inactive Jak2. This deletion construct co-immunoprecipitated with and inhibited the activity of the co-expressed kinase domain. This inhibition required the presence of the pseudokinase domain. Furthermore, complementation of a Jak2-deficient cell line with a Jak2 mutant lacking the pseudokinase domain deregulated IFN-γ signaling and caused constitutive ligand-independent activation of Stat1. These results suggest that the pseudokinase domain plays an important role in regulation of Jak2 activity in the absence of cytokine stimulation.

#1603 PKCζ INHIBITS GROWTH FACTOR INDUCED PHOSPHORYLATION AND ACTIVITY OF THE PKB/AKT REGULATOR OF CELL VIABILITY. M Mao, X -J Fang, Y Lu, B Cuevas, and G B Mills, *Univ of Texas MD Anderson Cancer Ctr, Houston, TX*

The PKB/AKT serine/threonine kinase located downstream of phosphatidylinositol 3-kinase (PI3K), is a major regulator of cellular survival. This anti-apoptotic activity is mediated, at least in part, by the regulation of BAD, caspase-9, GSK3α/β, forkhead and p70S6Kinase. PKCζ, which is also located downstream of PI3K, has previously been shown to inhibit PDGF-induced activation of PKB. To determine whether PKCζ may regulate signaling through the PI3K pathway, we explored the effect of overexpression of PKCζ and inhibitors of PKC on phosphorylation and activity of AKT. Following transfection, epitope-tagged PKCζ and PKB could be coprecipitated from the breast cancer cell line BT-549, indicating that PKCζ physically associates with PKB. The association of PKCζ and PKB was increased following EGF stimulation. EGF treatment resulted in an increase in phosphorylation of PKB on Ser473 and an increase in PKB activity. This increased phosphorylation and activity of PKB was reversed by expression of PKCζ. PKCζ did not reverse basal PKB phosphorylation suggesting that activation of PKCζ following EGF treatment is required for this function of PKCζ to be manifest. Compatible with a role of PKC in regulation of PKB phosphorylation and activity, the potent PKC inhibitor Ro-31-8220 induced phosphorylation and increased the activity of PKB. This effect of Ro-31-8220 was associated with a translocation of phosphorylated PKB to the cell membrane and of PKCζ from the cell membrane to the cytosol compatible with PKCζ being the target of Ro-31-8220. Thus PKCζ and potentially other PKC isozymes inhibit EGF mediated PKB phosphorylation and activation consistent with a generalized role in limiting growth factor mediated signaling through PKB.

#1604 THE PHOSPHOINOSITIDE 3-OH KINASE/AKT2 PATHWAY AS A CRITICAL TARGET FOR FARNESYLTRANSFERASE INHIBITOR-INDUCED APOPTOSIS. Jin Q Cheng, K. Jiang, D. Coppla, N. C Crespo, S. V Nicosia, A. D Hamilton, and S. M Sebti, *Univ of South Florida, Tampa, FL, and Yale Univ, New Haven, CT*

Farnesyltransferase inhibitors (FTIs) represent a novel class of anticancer drugs that exhibit a remarkable ability to inhibit malignant transformation without toxicity to normal cells. However, the mechanism by which FTIs inhibit tumor growth is not well understood. Here, we demonstrate that FTI-277 inhibits phosphoinositide 3-OH kinase (PI 3-kinase)/AKT2 mediated growth factor- and adhesion-dependent survival pathways and induces apoptosis in human cancer cells that overexpress AKT2. Furthermore, overexpression of AKT2, but not oncogenic H-Ras, sensitizes NIH 3T3 cells to FTI-277; and a high serum level prevents FTI-277-induced apoptosis in H-*Ras*- but not *AKT2*-transformed NIH 3T3 cells. A constitutively active form of AKT2 rescues human cancer cells from FTI-277-induced apoptosis. FTI-277 inhibits IGF-1-induced PI 3-kinase and AKT2 activation and subsequent phosphorylation of the pro-apoptotic protein BAD. Integrin-dependent activation of AKT2 is also blocked by FTI-277. Thus, a mechanism for FTI inhibition of human tumor growth is by inducing apoptosis through inhibition of PI 3-kinase/AKT2-mediated cell survival and adhesion pathway.

#1605 MAP KINASES IN PROSTATE CANCER. Aarti R Uzgare, and N. M Greenberg, *Baylor Coll of Medicine, Houston, TX*

We have previously demonstrated that specific changes in growth factor signaling correlate with the genesis and progression of prostate cancer in the autochthonous transgenic mouse model-TRAMP. In this study, we have characterized the expression and phosphorylation state of 3 members of the MAPK family, namely the Erks, Jnks and p38-MAPKs during the progression of prostate cancer in the TRAMP model. These MAPK molecules are known to integrate multiple signaling pathways as well as regulate processes central to tumorigenesis, such as proliferation, survival and migration. As a result of these studies, we have determined that P38MAPK is activated in prostatic intraepithelial neoplasia (PIN) as well as in moderately and well differentiated adenocarcinomas. Immunohistochemistry localized phospho-p38MAPK exclusively to the prostatic epithelium rather than the stromal compartment. Furthermore the activated p38MAPK was found in the cytoplasmic and nuclear compartments of PIN samples and in the nuclear compartment of well and moderately differentiated adenocarcinomas. In contrast, P38MAPK was not found to be activated in poorly differentiated adenocarcinomas obtained from intact or castrated TRAMP mice nor in metastatic deposits in the lymph nodes. Phospho-P38MAPK was completely absent in late stage tumors and metastases. These results suggest that the expression and activation of p38MAPK plays an important role in the initiation and/or early progression of prostate cancer and furthermore demonstrates that loss of activated p38 may serve as a novel diagnostic marker of prostate cancer progression.

#1606 AIK, A NOVEL ANDROGEN-INDUCIBLE KINASE, IDENTIFIED BY TYROSINE KINASE DISPLAY OF PROSTATE CANCER CELLS. Liang Xia, Dan Robinson, Hua-Chien Chen, Ai-Hong Ma, and Hsing-Jien Kung, *Nhri, Taipei, Taiwan, and UC Davis Cancer Ctr, Sacramento, CA*

Our laboratory is interested in studying tyrosine kinases and signal transduction pathways involved in prostate growth. We have developed an effective tyrosine kinase display method that allows us to describe all or nearly all tyrosine kinases using a single gel with a single RT-PCR reaction. The complete tyrosine kinase profiles of several widely used prostate cancer cell lines were obtained. This method, with its exquisite sensitivity, also permits the identification of kinases differentially expressed in prostate cancer cells, treated with or without hormone. We report here the identification of a novel, androgen-inducible kinase (AIK), which is modulated by androgen at the transcriptional level. Northern blot analysis revealed that AIK is specifically expressed in testis but not in other normal tissues examined. This male associated kinase contains sequence motifs related to both CDK and MAPK, and intriguingly, is localized in the nucleus. A tantalizing hypothesis is that AIK may be a downstream kinase, targeted by androgen action and serves to transmit androgen signal. Experiments are underway to define the signal pathways. 1. Robinson, D. et al, (1996), Proc Natl Acad Sci U S A 93(12), 5958-5962

#1607 RHOA-INDUCED MURINE PROSTATE CANCER CELL GROWTH IS MEDIATED BY PHOSPHATIDYLINOSITOL 3-KINASE (PI3K). Paramita M Ghosh, Marissa L Moyer, Roble Bedolla, Margarita Mikhailova, and Jeffrey I Kreisberg, *UTHSCSA, Audie Murphy Veterans Admin Hosp, San Antoino, TX*

To study the role of RhoA small GTPase in the growth of prostate tumor cells derived from transgenic mice with adenocarcinoma of the prostate (TRAMP cells), we developed cell lines stably expressing constitutively active RhoA(Q63L) (RhoA(Q63L) cells) or dominant-negative RhoA(T19N) (RhoA(T19N) cells) mutant proteins. RhoA(Q63L) cells displayed enhanced actin stress fiber assembly and grew at a faster rate than untransfected cells while the RhoA(T19N) cells displayed decreased growth and decreased actin stress fiber assembly. Myosin light chain (MLC) was highly phosphorylated in RhoA(Q63L) cells but not in the RhoA(T19N) cells. Treatment of RhoA(T19N) cells with the protein phosphatase 1 inhibitor okadaic acid resulted in actin stress fiber assembly and cell proliferation while treatment of RhoA(Q63L) cells with ML-9, a specific inhibitor of MLK kinase, induced growth arrest and actin stress fiber disassembly. This suggests that RhoA's effect on proliferation is mediated by actin stress fiber assembly. FACS analysis revealed that the growth of TRAMP and RhoA(Q63L) cells was inhibited by the PI3K inhibitor LY 294002 and not by PD 98059, an inhibitor of the mitogen activated protein kinase (MAPK) activator, MAPKK or MEK. RhoA(T19N) cells, on the other hand, were growth inhibited by PD 98059 and not by LY 294002. In addition, in cells expressing active RhoA, focal adhesion kinase (FAK) was activated and associated with PI3K, while in the absence of active RhoA, FAK/c-Src complexes were observed. Furthermore, cells which displayed active RhoA (TRAMP and RhoA(Q63L) cells) demonstrated activation of p70S6kinase while RhoA(T19N) cells showed activation of Akt. This suggests that RhoA induced cell proliferation is mediated by PI3K activation of p70S6kinase while in the absence of active RhoA, FAK/c-Src association results in activation of the Ras/MAPK signaling pathway for cell proliferation, as well as the PI3K cell survival pathway (activated Akt).

#1608 THE ROLE OF MITOGEN ACTIVATED PROTEIN KINASE PHOSPHATASE-1 IN SPHINGOLIPID MEDIATED INHIBITION OF APOPTOSIS IN C3H10T1/2 CELLS. S Sianna Castillo, and Dorothy Teegarden, *Purdue Univ, West Lafayette, IN*

Mitogen activated protein kinase (MAPK) phosphatase-1 (MKP-1) dephosphorylates and downregulates members of the MAPK family. Ceramine, a non-metabolizable analogue of the sphingolipid ceramide, stimulates apoptosis via stress activated protein kinase (SAPK), a MAPK family member. In the current study, the role of MKP-1 in the inhibition of apoptosis by a ceramide metabolite, sphingosine-1-P (SSP), was examined in C3H10T1/2 cells (10T1/2). Initially, 10T1/2 cells were treated with 10 μM C8 ceramide (Cer), 10 μM C8 ceramine (CN), 5 μM SSP, or a combination (CB) of 10 μM CN and 5 μM SSP for 12 hours and apoptosis was assessed. CN treatment induced a 7 fold increase in apoptosis compared with vehicle control. SSP inhibited CN mediated apoptosis as CB

15

Tyrosine Kinases and Cellular Signaling in Prostate Cancer

Hsing-Jien Kung, PhD, Clifford G. Tepper, PhD, and Ralph W. deVere White, MD

1. INTRODUCTION

There is very strong evidence that tyrosine kinases are involved in the growth and metastasis of prostate cancer *(65,152,165)*. Tyrosine kinases also play key roles in modulating tumor sensitivity to radiation- and chemical-induced apoptosis. Thus, there is hope that they may play an important role in the response of metastatic prostate cancer to hormonal intervention as well as to other chemotherapeutic approaches *(78)*. Their potential importance as targets for intervention is indicated by the FDA approval of the HER2/Neu-directed therapy, Herceptin, for breast cancer therapy and current clinical trials investigating its effectiveness for prostate cancer *(140)*. Presently, because of screening, 80% of prostate cancers are found while still localized to the gland. If we had the ability to determine which cancers would not metastasize, treatment could be given on an individual basis. Presently, prostate specific antigen (PSA) and tumor grade are the best markers we have. While being generally good clinical indicators, they lack specificity for the individual patient. There are a number of indications that tyrosine kinases may be valuable as prognostic markers in these situations *(65,152,165)*.

It has been estimated that there are about 1000–2000 protein kinases in the human genome; of these, 100 to 200 (i.e., 10%) are tyrosine kinases *(59)*. At present, there are 85 human tyrosine kinases identified in the GenBank database, and based on the relatively slow rate of discovery in the past few years, 100 is a better approximation to the total number of tyrosine kinases encoded by the human genome. In a given cell at a given stage, it is reasonable to assume that there are 30–50 tyrosine kinases expressed—a number large enough to provide characteristic tissue-specific patterns, but small enough to be identified in a simple screening. The hope for tyrosine kinases as prognostic markers rests with the fact that the identification of a stage-specific expression pattern will be identified in prostate cancer cells while they remain localized to the gland.

From: *Prostate Cancer: Biology, Genetics, and the New Therapeutics*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons

2. A TYROSINE KINASE PROFILE OF PROSTATE CANCER

In an effort to identify one or more novel biomarkers, an effective tyrosine kinase display approach was developed to identify all or nearly all tyrosine kinases expressed in prostate cancer using a single RT-PCR reaction and visualized in a single polyacrylamide gel. The approach takes advantage of common invariable motifs present in the catalytic domain of the great majority of tyrosine kinases (for example, DFG and DVW motifs in subdomains VII and IX, respectively). Degenerate primers based on reverse translation of these highly conserved sequence motifs are used to generate RT-PCR products of tyrosine kinases, and the resulting amplicons can be sequenced by traditional means—or better yet, subjected to restriction enzyme digestions so that the resulting fragments of different sizes reflect individual kinases. In the latter approach, the identities of the tyrosine kinases can be "read" directly from the gel, saving the time-consuming steps of cloning and sequencing. The band intensity corresponds well to the level of expression of a given kinase. When samples from normal and tumor tissues are compared, overexpressed tyrosine kinases can be readily identified. The first comprehensive tyrosine kinase profile was constructed from an androgen-sensitive, prostate specific antigen (PSA)-releasing prostate cancer xenograft CWR22. Table 1 summarizes the data derived from the display approach, as well as direct sequencing of the amplicons *(118)*. There are 20 receptor-tyrosine kinases and 12 nonreceptor tyrosine kinases. Among the receptor kinases, three (ErbB1, 2, and 3) come from the epidermal growth factor receptor (EGFR) family, and four (EphA1, A2, A4 and B4) from the Eph family. In addition to the Eph family of kinases, there are several cell adhesion molecule-related receptor kinases expressed in this CaP xenograft: Sky and Nyk, which carry neural cell adhesion molecule (NCAM)-like domains; the discoidin domain receptor tyrosine kinases Ddr1 and 2; and RET, which contains a cadherin domain. The presence of EGFR (ErbB1) nerve growth factor receptor (NGFR, trkA) *(39,49)*, fibroblast growth factor (FGFR) *(33,51,125,127)*, and insulin-like growth factor I receptor (IGFR) *(85,112)* are consistent with literature reports describing the responses of prostate cancer cells to these ligands. Among the nonreceptor tyrosine kinases represented, the src family contains three members (src, yes, and lck), and the related src-B family member Frk. The initial profile data also revealed several novel kinases, unknown at the time of discovery, but subsequently cloned: Nyk/Mer, an NCAM-related receptor tyrosine kinase *(53,80)*, and Etk/Bmx, a pleckstrin homology (PH) domain-containing tyrosine kinase *(114,144)*. The former has an elevated expression in CaP, compared to normal prostate epithelial cells, and the latter is expressed at a higher level in LNCaP than in other cell lines, and is implicated in IL-6 induced neuroendocrine differentiation. It is also noteworthy that trkA, trkC, and RET receptor kinases are expressed in CWR22 and LNCaP. This finding was initially surprising, as these kinases are known to be associated primarily with neuronal tissues, but is consistent with the idea that some of the prostate epithelial cells, especially when devoid of hormonal control, have neuroendocrine properties and can be transdifferentiated into such a lineage. RET has recently been shown to be overexpressed in high-grade CaP and high-grade PIN, but not in low-grade samples *(34)*. This finding suggests that RET may play a significant role in CaP progression, and raises the interesting possibility that high-grade CaPs are derived directly from high-grade PIN. Over-

Table 1
Tyrosine Kinase Profile in CWR22 CaP Xenograft

Receptor TK		Nonreceptor TK	
Family	Members	Family	Members
EGFR	ErbB1	*Src*	src
	ErbB2		yes
	ErbB3		lck
Eph	EphA1	*CSK*	Csk
	EphA2		
	EphA4	*Src-B*	Frk
	EphB4		
		JAK	JAK1
UFO/Axl	Sky/Tyro3		tyk2
	Nyk/mer		
		Abl	abl
Ddr	Ddr1		arg
	Ddr2		
		Btk	Etk/Bmx
PDGFR	PDGFR		
		FAK	FAK
FGFR	FGFR2		
	FGFR4	*ZAP70*	Syk
InR	IGFR		
MET	MET		
	Ron		
RET	RET		
NGFR	trkA		
	trkC		

all, the CWR22 tyrosine kinase profile described here is typical for all CaPs studied, although there is a greater similarity of the tyrosine kinase profiles between the two androgen sensitive models—CWR22 and LNCaP—than those of the androgen insensitive lines.

A number of ligands that transmit signals through receptor tyrosine kinases have been implicated in prostate cancer transformation and progression. This chapter focuses on the EGF receptor family of kinases and the signals transmitted by these receptors. The involvement of FGFR and NGFR families is briefly discussed to serve as a reference for further discussion. The literature citations are representative, and are not meant to be inclusive.

3. THE FIBROBLAST GROWTH FACTOR (FGF) RECEPTOR FAMILY OF TYROSINE KINASES

FGF-2 or basic FGF (bFGF), FGF-7 or keratinocyte growth factor (KGF), FGF-8, and FGF-9 are strong mitogens for prostate cells, and their production is associated with benign prostatic hyperplasia and CaP development *(35,52,71,103,121,146)*. Both FGF-2 *(142)* and FGF-7 *(160)* are provided by the stromal cells under normal conditions, but

FGF-2 expression in prostate epithelial cells is downregulated by androgen *(131)*. However, during the development of prostate cancer, alternative splicing of the FGFR2 locus leads to a switch in the expression of FGF receptor 2 isoforms from FGFR2(IIIb) to FGFR2(IIIc), with a concomitant shift in affinity from FGF-7 to FGF-2. An important event following this is the upregulation of FGF2 expression resulting in the establishment of an autocrine loop *(160)*, rendering the cells stromal-independent and androgen-independent *(17)*. In general, the FGF family of growth factors is viewed as progression factors for CaP. The observation that FGF-2 expression is regulated by androgen in prostate epithelial cells suggests that the molecular events leading to hormonal independence may occur at a much earlier stage than presently thought, and further implicates it as a critical factor to consider in relation to androgen ablative therapy *(131)*.

4. THE NERVE GROWTH FACTOR (NGF) RECEPTOR FAMILY OF TYROSINE KINASES

NGF has two receptors: the high-affinity receptor, trkA, and the low-affinity gp75NGFR. TrkA is a tyrosine kinase that serves to transduce NGF-induced differentiation and survival signals, whereas gp75NGFR tends to induce apoptosis. In the normal prostate, gp75NGFR is expressed in the epithelial cells, whereas the ligand NGF is expressed in the stroma *(109)*. The expression of gp75NGFR is reduced in prostate carcinomas and is completely absent in malignant CaP cell lines, Tsu-pr1, DU145, PC3, and LNCaP. Thus, there is an inverse correlation of expression of the gp75NGFR and CaP development. Consistent with its negative role in CaP progression is the finding that artificial expression of gp75NGFR in the Tsu-pr1 cell line results in NGF-induced apoptosis *(108)*. In contrast to the low-affinity receptor, trkA seems to be expressed in the majority of CaPs and all four CaP cell lines, and is a positive growth modulator for CaPs. NGF treatment of these cells stimulates their growth *(6)* and inhibitors of the NGF/trkA pathway inhibit CaP growth *(38,50)*. It is interesting that while NGF/trkA-induced signaling in neuronal cells results in neuronal differentiation, neuroendocrine differentiation in CaP is induced by agents such as interleukin-6 (IL-6) and forskolin, but not by NGF *(6,102)*. NGF also fails to induce the growth of normal prostate cells, and a recent finding suggests that the difference in the biological behaviors between tumor and normal cells cannot be attributed to mutations of trkA *(50)*, but more likely to the presence or absence of gp75NGFR. In two other studies, it was shown that NGF induces the invasiveness of DU145 *(49)* and hormone independence of Tsu-pr1 *(39)*. Thus, NGF plays a dual role in prostate cancer, depending on the repertoire of the receptors present in the cells: trkA behaves as a positive regulator for growth and tumor progression, whereas p75NGFR acts as an apoptosis inducer.

5. THE ErbB/EGF-RECEPTOR FAMILY OF TYROSINE KINASES

Among receptor kinases, the ErbB/EGF-receptor family is most frequently implicated in human malignancies. There are four members in this family—ErbB1, ErbB2, ErbB3, and ErbB4 *(25,73,110)*. The majority of prostate carcinomas express ErbB1, ErbB2, and ErbB3, but little or no ErbB4 *(118)*. ErbB1 is the EGF receptor, and frequently has been found overexpressed in tumors of epithelial origin. Amplification of ErbB1/EGFR has not been detected in CaP, but overexpression of this receptor is com-

mon. In nearly all CaP cell lines or tissues surveyed, an autocrine loop of TGF-α/EGF and ErbB1/EGFR exists, thus replacing the requirement for the normal stromal-derived ligand *(23,84,94,132,147)*. Inhibition of ErbB1/EGFR autocrine loop or the kinase activity of the receptor prevents the growth of CaP cells, indicating an essential role of ErbB1/EGFR in their growth *(12,118)*. Interestingly, such an inhibition also affects the actions of IGF-I and protein kinase A (PKA), indicating a general role for ErbB1/EGFR signaling in CaP growth *(112)*.

ErbB2, also called Neu (for the rat homolog) or HER2 (Human EGF Receptor 2), is the second member of this family, and figures prominently in human malignancies. The ErbB2 gene is amplified and overexpressed in 20–30% of primary breast cancers, and correlates with a poor prognosis. However, a humanized mouse monoclonal antibody (MAb) against ErbB2/HER2—Herceptin—exploits this feature as a novel molecular target, and has shown promise in clinical trials as an anti-breast cancer therapeutic agent, alone or in combination with standard chemotherapeutics *(104)*. Unlike breast carcinoma, genomic amplification of ErbB2 is rarely observed in prostate cancer *(13,45,74,88,153)*, but there are noteworthy exceptions *(122–124,134)*. The expression of both ErbB2 and 3 is either low or undetectable on normal prostate luminal epithelial cells, but is prevalent in prostate adenocarcinoma *(82)*. Accordingly, the expression of ErbB2 is considered to be an early event of CaP transformation *(96)*. The level of ErbB2 expression does not seem to vary significantly among CaPs of different histological grade *(57,76)*, although overexpression of ErbB2 in primary prostatic tissue predicts poor survival *(46,93)*. In addition, elevated serum levels of ErbB2 seem to correlate with progression of the disease status *(5a,93a)* and association of ErbB2 overexpression with the occurrence of metastatic disease has also been reported *(122)*. Further strong evidence for ErbB2 as an important factor in CaP metastasis was provided experimentally by the demonstration that in vitro transfection of rat prostatic epithelial cells with an oncogenic ErbB2 mutant (i.e. *Neu* mutation) resulted in metastatic tumors after orthotopic injection into nude mice *(87,166)*.

ErbB3, the third family member, is a kinase-impaired receptor, and requires dimerization with other family members to become an active signal transducer *(58)*. ErbB3 is expressed in the majority of primary and metastatic CaPs *(55,77,96,111,118)*. The ligand for ErbB3—heregulin (HRG) or neuregulin (NRG)—is reported to be expressed in 36% of CaPs analyzed by Leung et al. *(77)*, and the autocrine loop of HRG/ErbB3 appears to be associated with less favorable prognosis in advanced CaPs. By contrast, Lyne et al. *(82)* and Grasso et al. *(55)* found that HRG expression was absent in CaP specimens, three established CaP cell lines (LNCaP, DU145, PC3), and one xenograft (CWR22). However, HRG is expressed in an immortalized, nontumorigenic prostate epithelial cell line *(55)*, and is expressed in 100% of stroma, 100% of basal epithelial cells, and 58% of luminal cells in normal and benign hyperplastic prostatic tissue *(82)*. The latter studies suggest a downregulation of HRG and a concomitant loss of this autocrine loop during tumor progression, consistent with its growth arrest and differentiation effect on CaP cell lines.

In the next section we discuss the signaling events elicited by growth factors such as TGF-α/EGF and HRG, and by cytokines such as IL-6, as they pertain to CaP biology. Depending on the partners, and the individual receptors they associate with, they channel very different signals, with profoundly different biological outcomes.

6. TYROSINE KINASE SIGNALS THROUGH GROWTH FACTOR RECEPTORS

6.1. EGF/TGF-α Signals: Growth, Androgen Independence, Survival, and Invasion

Among the peptide growth factors, the action of EGF and TGF-α on prostate growth have been most extensively analyzed. There is a preponderance of evidence suggesting the involvement of EGF/TGF-α in the growth of prostate epithelial cells, and the autocrine loop of TGF-α/EGFR found in virtually all prostate cancer cells plays a significant part in their uncontrolled growth. Addition of EGFR-blocking antibody or specific inhibitors of the EGFR kinase *(12,167)* diminishes the growth of CaP. Inclusion of exogenous EGF and TGF-α in growth media further increases the growth rate of the prostate cancer cells, and this effect is synergistic with androgen. In the CWR22 xenograft model, the conversion from androgen-sensitive to the relapsed form (CWR22R) correlates with increased expression of TGF-α, indicating that the TGF-α/EGFR autocrine loop may override the requirement for androgen *(95)*. EGF/TGF-α stimulation of LNCaP induces tyrosine phosphorylation of EGFR/ErbB1, ErbB2, and ErbB3, with ErbB1 being the strongest. Homo and heterodimer formation of ErbB1/ErbB1, ErbB1/ErbB2, and ErbB1/ErbB3 dimers are all detected. Functionally, however, it appears that the ErbB1/ErbB1 homodimer is the most important. Using a LNCaP cell line where ErbB2 is functionally knocked out by the transfection of a single-chain antibody gene directed against ErbB2, it was shown that ErbB2 is dispensable for most of the EGF/TGF-α induced growth phenotypes *(54)*. Under these conditions, phosphorylation of ErbB3 is significantly reduced, indicating that ErbB2 mediates ErbB3 phosphorylation. Since only the growth properties of the ErbB2 "knockout" cells were studied, the role of ErbB2 and ErbB3 in other EGF-induced functions such as migration or survival have yet to be defined.

Intracellular signals are transmitted from membrane-associated tyrosine kinases to serine kinases or lipid kinases, and eventually to transcriptional factors through phosphorylation cascades. ErbB1/EGFR signals through several pathways in prostate cancer cells: Shc/mitogen-activated protein kinase (MAPK) *(18,56,112)*, phosphatidylinositol 3-kinase (PI3K)/Akt, phospholipase C-γ (PLC-γ)/protein kinase C (PKC), p21-activated kinase (PAK)/Jun N-terminal kinase (JNK), and the signal transducers and activators of transcription (STATs) *(55)*. The prevailing model is that upon ligand binding, homo- or heterodimers of the cognate receptors are formed, leading to transphosphorylation and activation of the intrinsic kinase activity. The active kinase is phosphorylated at the tyrosine residues which serve as anchor sites for a number of substrates with *src* homology 2 (SH2) and phosphotyrosine binding (PTB) domains, resulting in the phosphorylation of these substrates. Different substrates define the engagement of different pathways, although there is strong evidence that these pathways are interconnected and tend to modulate one another. A combination of the signal outputs from individual pathways defines the eventual phenotypes of the receptor activation. In subsequent sections, we review what is known about the signals involved in the growth, hormone-independent growth, survival, and motility induced by EGF/TGF-α. Except in cases of motility and invasion, where DU145 is a better model, the experi-

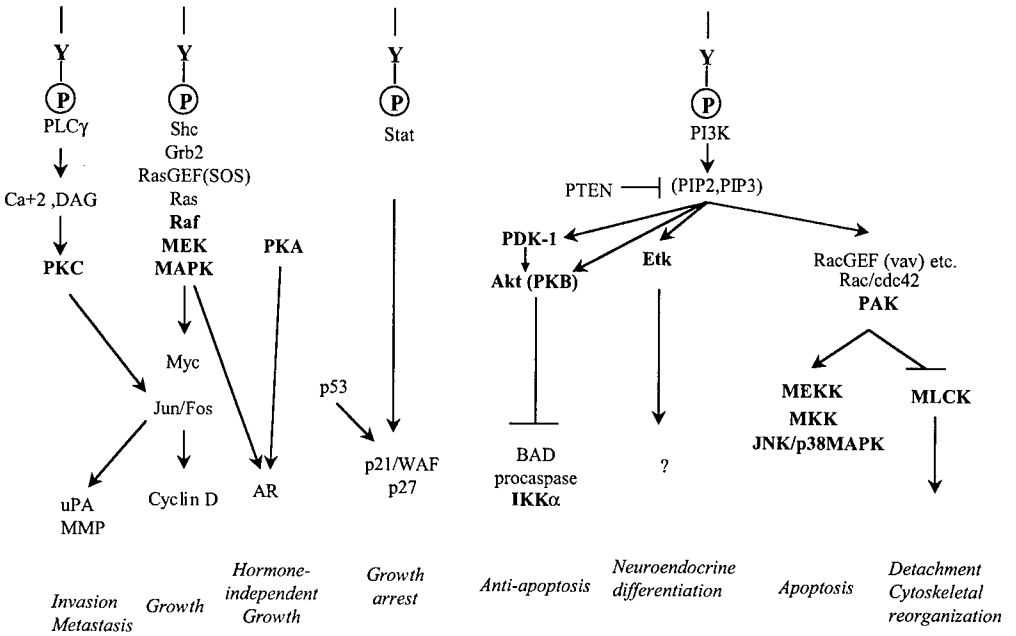

Fig. 1. Summary of the putative signal transduction pathways of prostate cancer cells initiated by tyrosine phosphorylations. Arrows indicate activation of downstream substrates. T-shaped bars indicate inactivations. The highlighted molecules are tyrosine or seirine kinases. The nomenclatures are described in the text. The data is based primarily on the studies of the erbB family of kinases.

mental data were principally derived from LNCaP studies. A summary diagram of the various signal transduction pathways is shown in Fig. 1.

6.2. *The Growth Signals*

Among the various pathways listed above, MAPK seems to be the most important in channeling the growth signals and the initial transformation of the cells. An early clue that attests to the importance of this pathway for growth and transformation comes from the fact that both *ras* and *raf*, which lie upstream in the pathway, are potent oncogenes and growth stimulators in a variety of cell types. In prostate cancer cells, mutations of *ras* or *raf* are rarely found, yet heightened activation of MAPK is detected in high-grade and hormone-independent CaP *(18)*. The TGF-α/ErbB autocrine loop found in most of the advanced CaP almost certainly contributes to the persistent activation of MAPK. Other overexpressed tyrosine kinases, such as RET and NYK, may also play a significant role. These receptors presumably activate MAPK through the well-defined Shc/Grb2/SOS/*ras*/*raf*/MEK pathway (Fig. 1) *(32,42,75,81,106,151)*. Although there are several upstream activators of MAPK, how MAPK drives the growth pathway is still not entirely clear. It has been reported that MAPK is able to phosphorylate c-Myc and Elk. Elk—an Ets-like transcriptional factor—is known to augment the expression of the AP-1 complex components fos, jun, and jun B. Both the AP-1 complex and c-Myc are known to activate cyclin D, thus propelling cells toward *S* phase.

Furthermore, Chen et al. recently showed that ectopic overexpression of cyclin D stimulates constitutive growth and tumorigenicity of LNCaP *(20)*. Perry et al. demonstrated that EGF activates cyclin D1 in LNCaP *(107)*, lending support to the above model. The same group further demonstrated that EGF-induced activation of cyclin D1 expression was dependent upon PKC. This finding seems reasonable, since EGF is known to activate PLC-γ, which in turn induces intracellular calcium elevations and produces diacylglycerol (DAG), two agonists for PKC, which is an activator of AP-1 complex. Activation of the AP-1 complex by the MAPK and PKC pathways follows two different phosphorylation cascades which are expected to be synergistic. It seems reasonable to propose that the elevated expression of cyclin D1—because of the combined action of MAPK and PKC pathways—plays an important role in EGF/TGF-α induced growth of LNCaP. It is noteworthy that LNCaP has wild-type p53 and Rb genes, making the overexpression of cyclin D1 necessary to overcome the actions of these cell-cycle gatekeepers. However, LNCaP carries a mutant PTEN which encodes a phosphatase specific for the product of PI3K, phosphatidylinositol 3,4,5-trisphosphate *(150)*. Deficiency of PTEN is believed to augment PI3K activity, which confers survival through Akt activation. However, at least one recent report implicates PTEN in G1 growth arrest and points to an unrecognized function of Akt in cell cycle progression *(117)*. This indicates that PTEN mutation may also contribute to the aggressive growth properties of LNCaP, although the mechanism is less clear.

6.3. The Survival Signals

The phosphatidylinositol 3-kinase pathway has recently attracted a great deal of attention because of its diverse effects. PI3K is a lipid kinase which catalyzes reactions to engender 3′-phosphoinositides (Fig. 1). These lipid moieties bind a class of molecules bearing PH domains, which translocate them to the cytoplasmic membrane and often alter their conformation. Akt/protein kinase B (PKB), a serine/threonine kinase involved in antiapoptosis, is one such substrate whose translocation to the membrane allows it to be phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1 (PDK1), another PH domain containing serine/threonine kinase activated by PI3K. A direct inhibitory effect on the cell death machinery has been demonstrated by phosphorylation of Bad *(31,37)* and procaspase-9 by active Akt *(15)*, inactivating their proapoptotic functions. Recruitment of the PI3K/Akt pathway can also have a cytoprotective effect via activation of nuclear factor-kappa B (NF-κB) and subsequent upregulation of an antiapoptotic transcriptional program *(101,120)*. Akt can mediate degradation of the NF-κB inhibitor IκB by interacting with, phosphorylating, and activating IκB kinase (IKK). IKK, in turn, phosphorylates IκB and targets it for degradation. NF-κB is then liberated and permitted to be translocated to the nucleus. This scheme is postulated to be the molecular basis of PI3K's ability to function as a survival factor. Recent studies by Lin et al. *(79)* and Carson and Weber *(16)* provide direct evidence that PI3K plays a significant role in sustaining survival of LNCaP, based on the observation that the PI3K inhibitors, wortmannin and LY294002, induce a high level of LNCaP cell death. These apoptotic effects can be partially rescued by treatment with EGF, which is known to activate PI3K—presumably via the activation of the ErbB1/ErbB3 heterodimer *(79)*. Interestingly, the Akt activity is not restored (since the

PI3K inhibitor is still present), and the authors postulate the existence of Akt-independent survival signals channeled by PI3K. ErbB3 carries multiple PI3K binding sites, and is particularly effective in forming a multimolecular complex with PI3K involving at least five additional tyrosine phosphorylated species *(54)*. It is conceivable that PI3K, with its multiple protein-protein interaction domains, may impart signal transduction by serving as an adaptor molecule without invoking lipid kinase activity.

Counteracting PI3K is the lipid phosphatase PTEN/MMAC1, originally discovered as a tumor suppressor gene for a number of cancers, including prostate cancers—up to 60% of which are defective in structure or expression of this gene *(150)*. This phosphatase removes a phosphate from the 3′ site of phosphatidyl polyphosphates, particularly phosphatidylinositol 3,4,5-trisphosphate, thereby diminishing the activating signal for Akt. Indeed, prostate cancers such as LNCaP lacking PTEN/MMAC1 have a constitutively high level of activated Akt *(155)*, which may account for the unusual durability of this cell line in harsh conditions. LNCaP, while growth-arrested, can survive long-term in serum-free and androgen-free conditions.

A new PI3K effector, Etk/Bmx, has recently been identified in prostate cancer cells *(114,118)*. Etk/Bmx is a tyrosine kinase that carries a PH domain at the N-terminus and belongs to the Btk family. Etk is the only member of the Btk family that is expressed in prostate cells such as LNCaP. In a manner similar to Akt, it was shown that Etk is able to protect CaP from thapsigargin or radiation-induced apoptosis *(159)*. While the molecular nature of this protective effect remains unclear, this finding demonstrates that there are other potential effectors of PI3K in antiapoptosis. STAT1,3, and 5 have also been shown to be phosphorylated and activated by Etk/Bmx *(128)*, and their connections to the protective effect of Etk are being examined.

6.4. The Hormone Independence Signals

LNCaP requires androgen for growth. The autocrine loop of TGF-α/EGFR existing in this cell line is apparently insufficient to override the hormone dependence. However, the addition of exogenous EGF or IGF-1 can induce LNCaP growth in the absence of synthetic androgen *(28)*. This suggests that either the EGF signal can activate the androgen receptor (AR) pathway in the absence of androgen (i.e., EGF and androgen in the same pathway), or EGF induces an independent growth pathway, obviating the need for androgen (i.e., EGF and androgen are in parallel pathways) or both. At least one report has indicated that the androgenic growth signal requires the interaction between amphiregulin and EGFR *(135)*, suggesting that the EGFR pathway lies downstream of the androgen pathway. Recent demonstrations that the MAPK pathway is able to activate androgen receptor transcriptional activity in the absence of its ligand *(1,27,162)* support this theory. While in the latter studies, the authors utilized overexpressed ErbB2 as a source for MAPK activation, ErbB2 is activated by EGF through heterodimerization with ErbB1, and as described here, EGF is a potent activator of MAPK. Thus, it is likely that EGF or TGF-α induced androgen-independent growth of LNCaP also follow the same pathways. In support of a role for ErbB2 in the conversion of prostate cancer cells into a hormone-independent state is the finding that prostatic acid phosphatase which diminishes ErbB2 activity restores hormone-sensitivity of a variant LNCaP line refractory to hormone induction *(90)*. If MAPK is the key

factor involved in activating AR in the absence of androgen, one would predict that MAPK agonists other than ErbB family members should also be able to convert hormone sensitive cells to refractory status—a theory which has not yet been tested. On the other hand, it is equally likely that MAPK is only one of the several pathways activated by ErbB1 or ErbB2 which contribute to AR activation. In the latter case, not all agonists that activate MAPK would induce AR independence. How does MAPK activate the transcriptional activity of AR? Chen et al. found that the target for the phosphorylation cascade is AR itself at a site where phosphorylation would strengthen the interaction with cofactors such as ARA50 or ARA70. These factors presumably enhance the DNA binding or transactivation function of the unliganded AR in a manner similar to the liganded AR. There is also evidence that PKA or elevated cAMP level activates AR in the absence of ligand *(126)*. While this is probably the result of direct phosphorylation of AR by PKA *(126)*, there are at least two reports indicating a synergy between PKA and ErbB1 in the activation of MAPK in LNCaP *(18,112)* which may contribute to the androgen independent activation of AR.

6.5. The Motility and Invasion Signals

EGF is known to induce cell motility, detachment, and invasion of cancer cells. As these processes are dependent upon the cell type and the extracellular matrix used, it is therefore difficult to generalize. For instance, EGF or TGF-α seems to have little effect on the cytoskeletal structure, motility, or invasiveness of LNCaP cells, although it promotes chemomigration of Tsu-pr1 cells *(116)* and motility of DU145 cells *(148)*. In the case of DU145, the activation of PLC-γ seems to be crucial in the migratory properties of the cell, presumably through the activation of PKC and the mobilization of calcium *(149)*. The same authors also showed that disassembly of focal adhesions—a step linked to migration—involves the MAPK pathway *(158)*. In other cell types, growth-factor-induced cell migration often involves the PI3K pathway and small GTPases such as rac1/rhoA/cdc42.

In addition to cell motility, invasion requires the release of proteinases to digest the extracellular matrix. In prostate cancer, EGF induces the release of matrilysin and urokinase (uPA), two molecules strongly implicated in the invasion process *(43,44,67, 115,143)*. The pathway leading to uPA activation involves AP-1, and thus probably includes MAPK and JNK as its effectors *(154)*.

7. HEREGULIN (HRG) SIGNALS: GROWTH ARREST, CYTOSKELETAL REORGANIZATION, AND APOPTOSIS

EGF and TGF-α are involved in many aspects of prostate cancer progression by engaging with ErbB family receptors, primarily ErbB1 and ErbB2. However, ErbB3 and ErbB4 are the high-affinity receptors for heregulin, a polypeptide ligand that has varying effects on different prostate cancer cell lines *(14)*. HRG was also identified as neuregulin (NRG) and Neu differentiation factor (NDF), among other names, but will be referred to as HRG in this chapter *(64,86,105)*. Since ErbB4 is not usually expressed in CaP, HRG functions in this cell type primarily through activation of ErbB3/ErbB2, and to a lesser extent, through ErbB3/ErbB1 heterodimers. Under these conditions, an ErbB3/ErbB3 homodimer may also form, but would be unproductive, as ErbB3 is

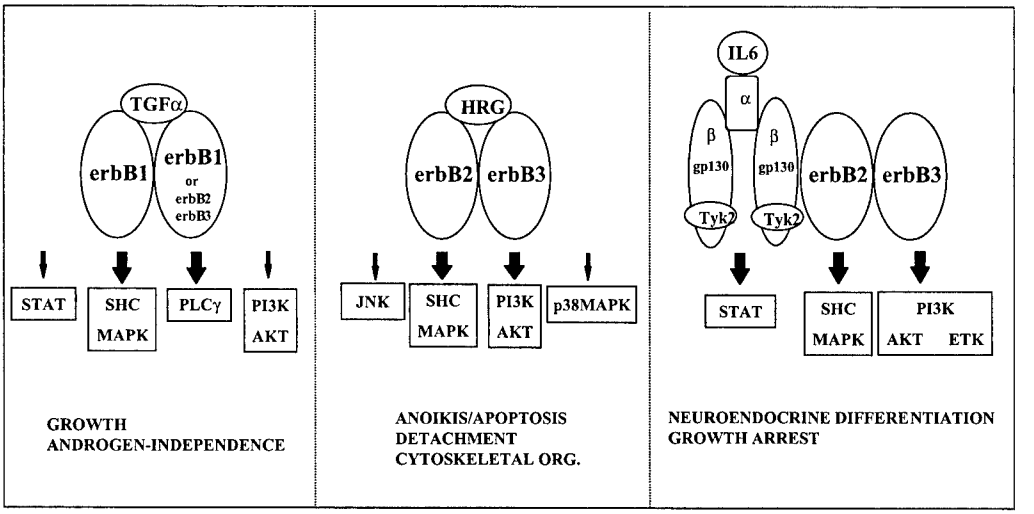

Fig. 2. Schematic representation of key ErbB-mediated signal transduction pathways induced by TGF-α, HRG, and IL-6 in LNCaP prostate cancer cells. Signaling is initiated by ErbB receptor heterodimerization induced by direct ligand binding (i.e., TGF-α, HRG) or indirectly via association with the activated IL-6 receptor. Activation of the intrinsic ErbB tyrosine kinase activity leads to tyrosine phosphorylation of intracytoplasmic domains, recruitment of proteins containing SH2 or PTB domains, and propagation of the signal. Well-characterized signaling pathways in this system are annotated in boxes and the relative strengths of each pathway are indicated by the thickness of the arrows.

kinase-impaired and requires other kinase-active receptors to transphosphorylate it. Indeed, using a LNCaP cell line that has ErbB2 knocked out by the ErbB2 antibody gene-trapping technique, the signaling ability of HRG is completely eliminated *(54)*. Thus, in prostate cancer cells, ErbB2 is a vital component of the HRG signal machinery, and the ErbB2/ErbB3 heterodimer is the principal component activated by HRG. Expression analysis of HRG provides an interesting contrast to that of EGF/TGF-α: HRG is highly expressed in normal prostate epithelial cells—especially basal cells and stromal cells—but at low or undetectable levels in prostate cancer cells *(55,82)*. None of the commonly used CaP cell lines express HRG, whereas an immortalized, normal prostate-epithelial cell line does (MLC-SV40). This suggests that NDF may serve a differentiation or antiproliferative role in prostate cancer cells, as it does in some of the breast cancer cell lines *(29,145)*.

Some of the intracellular signals such as MAPK—which are induced by HRG and by EGF/TGF-α—are similar, but others are different, and have distinct biological consequences. Some of the notable differences are summarized here (Fig. 2). For instance, after HRG treatment, PI3K is assembled into an "activation complex," which can be differentiated from that induced by EGF/TGF-α based upon the tyrosine-phosphorylated band patterns present in the complexes. In addition, HRG treatment activates p38MAPK and JNK, but not PLC-γ and STATs *(55)*. Akt is activated only moderately over the already high background of constitutive activity in LNCaP.

7.1. The Growth Arrest Signals

LNCaP grows well in media containing androgen and serum. In the presence of HRG, the growth rate declines, indicating that HRG transmits a dominant growth-arrest signal *(55,82)*. Addition of the MEK inhibitor PD98059 does not restore growth of LNCaP treated with HRG, nor does the addition of a PI3K inhibitor (LY294002). These results are somewhat difficult to interpret, because LY294002 induces apoptosis even in the absence of HRG. How does HRG induce growth arrest? Bacus et al. *(7)* demonstrated that HRG induces the expression of p53 and p21WAF1, a CyclinD/CDK2 inhibitor, in LNCaP. Yu et al. *(163)* also showed that ErbB2 overexpression upregulates the transcription of p21WAF1. The accumulation of p21 may be one reason that cells go into quiescence. Consistent with this concept is the finding that PDGF treatment of prostate cancer cells results in the induction of p21WAF1 expression, G1 arrest, and the sensitization toward radiation-induced apoptosis *(70)*. Additionally, tamoxifen-induced apoptosis of PC3 and DU145 apparently involves the upregulation of p21WAF1 expression *(119)*, as does cell-cycle inhibition of DU145 induced by type-I interferon alpha *(62)*. Thus, p21WAF1 may be a common mediator of growth arrest in prostate cancer cells.

7.2. The Cytoskeletal Reorganizaton and Detachment Signals

In addition to growth arrest, treatment of LNCaP with HRG induces immediate, cytoskeletal rearrangements characterized by the formation of filopodia, lamellopodia, and stress fibers. A distinct morphological change accompanies this by an alteration in cell shape to a more rounded appearance from the slender shape assumed by typical epithelial cells *(54)*. This is followed by detachment of cells from the plate. Specific inhibitor experiments have indicated that this process depends on PI3K rather than MAPK. The involvement of PI3K in shaping the cytoskeletal structure has been well-documented in other systems, and is thought to engage rac/rho/Cdc42 *(133)*. Rac and Cdc42 are small *ras*-like, RhoA family GTPases with an approximate molecular mass of 21 kDa, and are often referred to as p21 small G proteins. This family of GTPases is known to induce filopodia, lamellipodia, and the disassembly of stress fibers, counteracting the action of RhoA, which contributes to stress fiber assembly. Rac and Cdc42 are activated by guanine exchanger factors (GEF) such as vav, which contain PH domains and thus are effectors for PI3K *(2,83)*. This may explain why PI3K inhibition prevents cell-shape changes and detachment. Rac and Cdc42 activate several kinases, including p21-activated kinases (PAKs) *(137)*, which comprise a family of kinases that share homology in their kinase domains to yeast Ste-20-like kinases. At least three members have been cloned from mammalian cells. Interestingly, a dual role for PAK exists—it is involved in cytoskeletal reorganization and cell movement by phosphorylating myosin light-chain kinase *(30,130,136)*, and it is able to mediate apoptosis through the activation of JNK and p38 MAP kinases *(24,91)* (Fig. 1).

7.3. The Anoikis/Apoptosis Signals

The detached cells soon undergo apoptosis through a process referred to as anoikis *(47)*. It has been postulated that anoikis involves MEKK and either one of the stress-activated kinases, JNK or p38MAPK *(48)*, although an alternative mechanism may be

responsible *(69)*. The activation of JNK and p38MAPK in LNCaP by HRG, but not by EGF is consistent with this hypothesis. Apoptosis induced this way must be able to offset the antiapoptotic effect of Akt activity, which is constitutively present in this cell type. p38MAPK activation is known to induce differentiation and apoptosis in several cell types, thereby providing additional support for this as a mechanism of cell death in this system *(97,157,164)*. Perhaps most germane to the present discussion is the report that in the breast cancer cell line SKBR3, HRG induces apoptosis through p38 activation and subsequent apoptosis *(29)*.

8. TYROSINE KINASE SIGNALS THROUGH A CYTOKINE RECEPTOR

8.1. IL-6 Signals: Hormone Independence, Growth Arrest, and Neuroendocrine Differentiation

Few interleukins are implicated in CaP progression; one that has drawn considerable attention is IL-6. Originally identified as a regulator of immune and inflammatory responses, IL-6 has now been recognized as a key factor involved in the growth and metastasis of several types of neoplasms *(61)*. IL-6 has a two-component receptor—the p80 α-subunit which binds IL-6 and the gp130 β-subunit which is the actual signal transducer and is shared with other cytokine receptors such as oncostatin M (OM), leukemia inhibitory factor (LIF), and interleukin-11 (IL-11) *(60)*. Both components of the IL-6 receptor are expressed in all prostate cancer specimens and cell lines surveyed, indicating a role for IL-6 in CaP biology *(139)*. However, this role is complex. In androgen-dependent cells such as LNCaP, dihydrotestosterone (DHT) suppresses IL-6 expression through a mechanism of androgen receptor-mediated repression of NF-κB activity *(68)*. Addition of exogenous IL-6 to this cell line inhibits growth and induces neuroendocrine differentiation *(36,114)*. By contrast, IL-6 functions as a growth factor for androgen independent CaP cell lines, DU145 and PC3, increasing their growth rate *(100)* and colony-formation potential, and conferring resistance to certain chemotherapeutic agents and tumor necrosis factor (TNF)-mediated cell death *(9,21,98)*. The IL-6 autocrine loop is present in all androgen independent CaP cells, but not in dependent lines *(21)*, suggesting that the IL-6 autocrine loop may be functionally linked to the androgen independent phenotype. These observations, coupled with the finding that the level of circulating IL-6 is elevated in metastatic prostatic carcinoma *(3,4)* implicate IL-6 in CaP progression. While this view seems to be at odds with the antiproliferative effect of IL-6 in androgen dependent cells such as LNCaP, it should be noted that neuroendocrine differentition results in the release of neurotrophins which facilitate the survival and chemomigration of the surrounding CaP cells.

8.2. Hormone Independent Growth and Antiapoptosis Signals

IL-6 is a strong inducer of growth and survival of androgen-independent DU145 and PC3 *(10,11)*. Among the signals triggered by IL-6, MAPK is likely to be responsible for the growth response and PI3K for drug and apoptosis resistance, by analogy to the action of EGF. Since PC3 and DU145 do not express androgen receptors, the IL-6-induced growth and survival signals must pass through an androgen receptor independent pathway. Interestingly, if the androgen receptor status is artificially restored by transfection into DU145, IL-6 is able to stimulate androgen receptor dependent gene transcripton

(e.g., a reporter gene driven by the androgen-response element (ARE)) in the absence of androgen *(63)*. This could be inhibited by the nonsteroidal androgen-receptor antagonist bicalutamide (Casodex), indicating that IL-6 indeed mediates its activation through the androgen receptor. The authors have further demonstrated that this activation requires the activities of PKC, PKA, and MAPK. In the AR-positive cell line LNCaP, IL-6 is able to induce transcription from the PSA promoter in the absence of androgen (Dr. Li-Fen Lee, personal communication). Since PSA promoter activation is critically dependent upon AR activity, the experiment described serves as confirmation of the ability of IL-6 to activate the androgen receptor. This is an intriguing finding which suggests that IL-6 may facilitate the initial transition of a prostate cancer cell from hormone dependence to independence by acting as a pseudo-activator. Eventually, in the case of DU145 and PC3, androgen receptor expression is lost and the androgen receptor independent IL-6 pathway takes over. Since AR is known to suppress the transcription of the IL-6 gene *(68)*, the loss of AR further increases the expression of IL-6, setting up a permanent autocrine loop. Oncostatin M, which, like IL-6, activates gp130, is also capable of stimulating the growth of DU145 *(11)*. In this case, STAT3 activation is required. This suggests that although diverse ligands interacting with similar receptors leads to an identical phenotype, diversification of signal transduction pathways contributes to CaP tumor progression and increases the likelihood for selection of aggressive phenotypes.

8.3. The Growth Arrest and Neuroendocrine Differentiation Signals

In contrast to the strong growth stimulation of DU145 and PC3, IL-6 inhibits the growth of LNCaP and induces neuroendocrine (NE) differentiation *(114)*. The acquisition of cells with NE characteristics has been reported to be an early marker for development of androgen independence of prostate cancers, and tumor cell populations have been reported to become enriched for NE cells following long-term antiandrogen therapy *(22,40,99)*. It has been suggested that these NE cells function as a paracrine source of factors to support androgen independent growth of the surrounding cancer cells. NE cells are identified by neurite outgrowth, the presence of neurosecretory granules, and their ability to express a wide variety of neuronal-specific markers such as chromogranin A, neurospecific enolase, and a number of potentially mitogenic neuropeptide hormones, including parathyroid hormone-related peptide, bombesin, serotonin, calcitonin, and others *(26)*. Increased serum levels of chromograinin A are found to correlate well with the acquisition of androgen independence and CaP progression. Although NE cells are nonmitotic, proliferating carcinoma cells have been found in close proximity to them. These observations suggest that NE differentiation of prostate cells is associated with progression of CaP towards an androgen independent state. The origin of NE cells in CaP is not entirely clear, although it has been suggested that they are derived from either prostate stem cells or prostate epithelial cells through transdifferentiation. The fact that some prostate cancer cell lines, such as LNCaP, can undergo NE differentiation suggests that at least a subset of NE cells is derived from prostate epithelial cells. In addition to IL-6 *(114)*, a number of diverse stimuli have been described to induce NE phenotypes of prostate cancer cells. These include the cytokines IL-1 and IL-2 *(41)*, long-term serum/androgen starvation *(138)*, and eleva-

tion of intracellular cyclic AMP (cAMP) levels *(26)*. A variety of physiological and pharmacological agents can increase cAMP levels, such as epinephrine, forskolin (adenylate cyclase activator), and the cAMP analog dibutyryl cAMP *(8,26)*. Interestingly, withdrawal of forskolin and epinephrine from LNCaP cells induces the loss of neuritic processes and the reacquisition of a morphology typical of untreated cells, indicating that NE differentiation is reversible and that the affected cells were arrested in growth, but not terminally differentiated and senescent. In the IL-6-induced differentiation model system, treated LNCaP cells were shown to be growth arrested at the G1/S boundary *(92,114)*. This growth arrest apparently involves the activation of p27 at the transcriptional level, but is independent of SHP2 association with IL-6R *(72,92)*. Since it has been demonstrated that elevated cAMP levels can potentiate IL-6 signal transduction, cross-communication between the cAMP and IL-6 pathways may further augment NE differentiation *(19)*. An even more dynamic and aggressive environment can conceivably be established after malignant prostatic neuroendocrine begins secreting mitogenic neuropeptides, which can also contribute to cAMP elevation.

What are the signal pathways which lead to neuroendocrine differentiation of LNCaP cells? The studies of IL-6 *(114)* and cAMP agonists *(26)* have provided insight into this process. In the case of cAMP elevation, it is expected that PKA is involved, and indeed forced expression of a dominant-negative mutant of PKA blocks the differentiation process (M. Cox and S. Parsons, personal communication). PKA activates CREB and ATF, and these transcriptional factors are likely to be responsible for activating the genes involved in neuroendocrine differentiation. Yet, in LNCaP, PKA also activates the MAPK pathway via Rap1, which leads to jun/fos activation *(18)*. Thus, a combination of these bZIP proteins may be involved in this phenotype. The mechanism whereby IL-6 induces NE was more obscure. Early studies in PC12—a rat pheochromocytoma cell line which can be induced by IL-6 to undergo neuronal differentiation—revealed the role of the PI3K pathway in this process *(89,141,156,161)*. When LNCaP was treated with IL-6, the MAPK, JAK/STAT3 and PI3K pathways were all strongly activated *(114)* (Fig. 2). In addition, a PH-domain containing tyrosine kinase, Etk/Bmx, was also activated, and serves as an effector molecule for PI3K. This is understandable, since the PH domain interacts with 3′-phosphoinositides, metabolic products of PI3K *(129)*. By analogy to Btk and Itk, close cousins of Etk, 3′-phosphoinositide binding unfolds this family of proteins, exposing their kinase domains and translocating them to the cytoplasmic membrane where they can be activated by *src*-like kinases *(5)*. That Etk/Bmx is crucial for IL-6-induced neuroendocrine differentiation was demonstrated by the differentiation-resistant phenotype acquired by LNCaP cells stably transfected with a dominant-negative Etk expression construct *(114)*. A key question raised by this study was: What transmits the high level of tyrosine phosphorylation signal, considering that IL-6 receptor itself is not a tyrosine kinase? Jak family kinases known to be activated by cytokine are possible candidates. Tyk2 is indeed activated in IL-6 treated LNCaP cells, but more intriguingly, ErbB2 and ErbB3 are also activated *(113)* (Fig. 2). ErbB1—the only other receptor tyrosine kinase in this family expressed in LNCaP cells—is not activated, indicating the specificity of this cross-communication. Furthermore, ErbB2 forms a stable complex with the gp130 subunit of the IL-6 receptor in an IL-6 dependent manner. This suggests that IL-6 activation of ErbB kinases

occurs through direct engagement, and that ErbB2 and Tyk2 both contribute to the elevation of tyrosine phosphorylation induced by IL-6 in LNCaP. Using the ErB2-trapping approach described here (i.e., single-chain antibody expression construct) to functionally knock out ErbB2, it was shown that both ErbB3 and MAPK activation require ErbB2 engagement. This example illustrates how a cytokine receptor can diversify its signal through engagement with receptor tyrosine kinases, which may help explain the complex and pleiotropic phenotypes induced by IL-6. Consistent with a role for ErbB2 in neuroendocrine differentiation is the demonstration that prostatic neuroendocine cells with dendritic appearance and chromogranin A expression express ErbB2 *(66)*. These findings indicate that at least the PKA and PI3K/Etk pathways are involved in the neuroendocrine differentiation of LNCaP. Most likely, other pathways such as MAPK or STAT3 also contribute to this phenotype.

9. CONCLUSION

Tyrosine kinases play significant roles in cellular signaling in prostate cancer. They respond to growth factors as well as to cytokines. The widely recognized pathways such as MAPK, PI3K, PLC-γ, and STATs are activated by multiple inducers, and clearly participate in the signaling (Fig. 1). Yet these pathways do not tell the whole story, and the final outcome depends not only on the combination of multiple pathways, but on their relative intensities. By design, our studies have focused on signals mediated by ErbB kinases. Fig. 2 provides a summary of the various signal pathways outlined in this chapter. Whereas TGF-α/EGF is a strong growth stimulator for all CaP cells thus far studied, HRG induces anoikis and apoptosis. These two diametrically opposite phenotypes nevertheless share overlapping signals. For instance, both TGF-α and HRG activate SHC/MAPK (thick arrows) strongly and PI3K to varying degrees. By contrast, PLC-γ is solely induced by TGF-α, and p38MAPK by HRG. The latter signals thus may be responsible for the particular phenotypes induced by individual growth factors. A comparison of HRG and IL-6 signal pathways offers another intriguing scenario. Here, both involve an ErbB2/ErbB3 complex—one activated by HRG from outside and the other activated by complex formation with IL-6 receptor from within. Again, common signals associated with ErbB2 and ErbB3, such as MAPK and PI3K, are induced. Yet the biological consequences are profoundly different. The likelihood that the Tyk2/STAT pathway contributes to the unique neuroendocrine phenotype needs further examination. How and whether it is related to the cAMP agonist-induced neuroendocrine phenotype are also worth exploring. Prostate cancer offers a complex, yet fascinating biological system to dissect key signal molecules involved in cell fate determination. A full understanding of these pathways is important for scientific study, and may benefit strategies to modify the tumor behavior (e.g., to make tumors more prone to apoptosis and enhance sensitivity to therapy). The success of Herceptin may herald the use of antityrosine kinase antibodies or inhibitors as general anticancer agents. Future intervention strategies may also include potentiating the toxicity of Herceptin through a combination with heregulin, as well as disruption of autocrine growth factor/receptor loops. In light of recent findings, it will also be critical to consider the interplay of androgen receptor signaling with the pathways discussed in the design of tyrosine kinase-based therapies. For all these reasons, and to explore the

possibility of uncovering potential tumor markers, the study of tyrosine kinases and cellular signaling in prostate cancer promises to be a flourishing area of research in the new millennium.

REFERENCES

1. Abreu-Martin, M. T., A. Chari, A. A. Palladino, N. A. Craft, and C. L. Sawyers. 1999. Mitogen-activated protein kinase 1 activates androgen receptor-dependent transcription and apoptosis in prostate cancer. *Mol. Cell Biol.* **19:** 5143–5154.
2. Adam, L., R. Vadlamudi, S. B. Kondapaka, J. Chernoff, J. Mendelsohn, and R. Kumar. 1998. Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *J. Biol. Chem.* **273:** 28,238–28,246.
3. Adler, H. L., M. A. McCurdy, M. W. Kattan, T. L. Timme, P. T. Scardino, and T. C. Thompson. 1999. Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. *J. Urol.* **161:** 182–187.
4. Akimoto, S., A. Okumura, and H. Fuse. 1998. Relationship between serum levels of interleukin-6, tumor necrosis factor-alpha and bone turnover markers in prostate cancer patients. *Endocr. J.* **45:** 183–189.
5. Andreotti, A. H., S. C. Bunnell, S. Feng, L. J. Berg, and S. L. Schreiber. 1997. Regulatory intramolecular association in a tyrosine kinase of the Tec family. *Nature* **385:** 93–97.

5a. Arai, Y., T. Yoshiki, and O. Yoshida. 1997. c-erbβ-2 oncoprotein: a potential biomarker of advanced prostate cancer. *Prostate* **30:** 195–201.

6. Angelsen, A., A. K. Sandvik, U. Syversen, M. Stridsberg, and H. L. Waldum. 1998. NGF-beta, NE-cells and prostatic cancer cell lines. A study of neuroendocrine expression in the human prostatic cancer cell lines DU-145, PC-3, LNCaP, and TSU-pr1 following stimulation of the nerve growth factor-beta. *Scand. J. Urol. Nephrol.* **32:** 7–13.
7. Bacus, S. S., Y. Yarden, M. Oren, D. M. Chin, L. Lyass, C. R. Zelnick, et al. 1996. Neu differentiation factor (Heregulin) activates a p53-dependent pathway in cancer cells. *Oncogene* **12:** 2535–2547.
8. Bang, Y. J., F. Pirnia, W. G. Fang, W. K. Kang, O. Sartor, L. Whitesell, et al. 1994. Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc. Natl. Acad. Sci. USA* **91:** 5330–5334.
9. Borsellino, N., A. Belldegrun, and B. Bonavida. 1995. Endogenous interleukin 6 is a resistance factor for *cis*-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res.* **55:** 4633–4639.
10. Borsellino, N., A. Belldegrun, and B. Bonavida. 1995. Endogenous interleukin 6 is a resistance factor for *cis*-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res.* **55:** 4633–4639.
11. Borsellino, N., B. Bonavida, G. Ciliberto, C. Toniatti, S. Travali, and N. D'Alessandro. 1999. Blocking signaling through the Gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumor cells to etoposide and cisplatin-mediated cytotoxicity. *Cancer* **85:** 134–144.
12. Bos, M., J. Mendelsohn, Y. M. Kim, J. Albanell, D. W. Fry, and J. Baselga. 1997. PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin. Cancer Res.* **3:** 2099–2106.
13. Bubendorf, L., J. Kononen, P. Koivisto, P. Schraml, H. Moch, T. C. Gasser, et al. 1999. Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence *in situ* hybridization on tissue microarrays (published erratum appears in *Cancer Res.* 1999 Mar. 15;59(6):1388). *Cancer Res.* **59:** 803–806.

14. Burden, S. and Y. Yarden. 1997. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* **18:** 847–855.
15. Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, et al. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282:** 1318–1321.
16. Carson, J. P., G. Kulik, and M. J. Weber. 1999. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. *Cancer Res.* **59:** 1449–1453.
17. Carstens, R. P., J. V. Eaton, H. R. Krigman, P. J. Walther, and M. A. Garcia-Blanco. 1997. Alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) in human prostate cancer. *Oncogene* **15:** 3059–3065.
18. Chen, T., R. W. Cho, P. J. Stork, and M. J. Weber. 1999a. Elevation of cyclic adenosine 3′,5′-monophosphate potentiates activation of mitogen-activated protein kinase by growth factors in LNCaP prostate cancer cells. *Cancer Res.* **59:** 213–218.
19. Chen, T., R. W. Cho, P. J. Stork, and M. J. Weber. 1999b. Elevation of cyclic adenosine 3′,5′-monophosphate potentiates activation of mitogen-activated protein kinase by growth factors in LNCaP prostate cancer cells. *Cancer Res.* **59:** 213–218.
20. Chen, Y., L. A. Martinez, M. LaCava, L. Coghlan, and C. J. Conti. 1998. Increased cell growth and tumorigenicity in human prostate LNCaP cells by overexpression to cyclin D1. *Oncogene* **16:** 1913–1920.
21. Chung, T. D., J. J. Yu, M. T. Spiotto, M. Bartkowski, and J. W. Simons. 1999. Characterization of the role of IL-6 in the progression of prostate cancer. *Prostate* **38:** 199–207.
22. Cohen, R. J., G. Glezerson, and Z. Haffejee. 1991. Neuro-endocrine cells-a new prognostic parameter in prostate cancer. *Br. J. Urol.* **68:** 258–262.
23. Connolly, J. M. and D. P. Rose. 1990. Production of epidermal growth factor and transforming growth factor-alpha by the androgen-responsive LNCaP human prostate cancer cell line. *Prostate* **16:** 209–218.
24. Coso, O. A., M. Chiariello, J. C. Yu, H. Teramoto, P. Crespo, N. Xu, et al. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81:** 1137–1146.
25. Coussens, L., T. L. Yang-Feng, Y. C. Liao, E. Chen, A. Gray, J. McGrath, et al. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* **230:** 1132–1139.
26. Cox, M. E., P. D. Deeble, S. Lakhani, and S. J. Parsons. 1999. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. *Cancer Res.* **59:** 3821–3830.
27. Craft, N., Y. Shostak, M. Carey, and C. L. Sawyers. 1999. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase (see comments). *Nat. Med.* **5:** 280–285.
28. Culig, Z., A. Hobisch, M. V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, et al. 1994. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.* **54:** 5474–5478.
29. Daly, J. M., M. A. Olayioye, A. M. Wong, R. Neve, H. A. Lane, F. G. Maurer, et al. 1999. NDF/heregulin-induced cell cycle changes and apoptosis in breast tumour cells: role of PI3 kinase and p38 MAP kinase pathways. *Oncogene* **18:** 3440–3451.
30. Daniels, R. H., P. S. Hall, and G. M. Bokoch. 1998. Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. *EMBO J.* **17:** 754–764.
31. Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, et al. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:** 231–241.

32. Davis, R. J. 1995. Transcriptional regulation by MAP kinases. *Mol. Reprod. Dev.* **42:** 459–467.
33. Davol, P. A. and A. R. J. Frackelton. 1999. Targeting human prostatic carcinoma through basic fibroblast growth factor receptors in an animal model: characterizing and circumventing mechanisms of tumor resistance. *Prostate* **40:** 178–191.
34. Dawson, D. M., E. G. Lawrence, G. T. MacLennan, S. B. Amini, H. J. Kung, D. Robinson, et al. 1998. Altered expression of RET proto-oncogene product in prostatic intraepithelial neoplasia and prostate cancer (see comments). *J. Natl. Cancer Inst.* **90:** 519–523.
35. De Bellis, A., C. Crescioli, C. Grappone, S. Milani, P. Ghiandi, G. Forti, et al. 1998. Expression and cellular localization of keratinocyte growth factor and its receptor in human hyperplastic prostate tissue. *J. Clin. Endocrinol. Metab.* **83:** 2186–2191.
36. Degeorges, A., R. Tatoud, F. Fauvel-Lafeve, M. P. Podgorniak, G. Millot, P. de Cremoux, et al. 1996. Stromal cells from human benign prostate hyperplasia produce a growth-inhibitory factor for LNCaP prostate cancer cells, identified as interleukin-6. *Int. J. Cancer* **68:** 207–214.
37. del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera, and G. Nunez. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278:** 687–689.
38. Delsite, R. and D. Djakiew. 1996. Anti-proliferative effect of the kinase inhibitor K252a on human prostatic carcinoma cell lines. *J. Androl.* **17:** 481–490.
39. Delsite, R. and D. Djakiew. 1999. Characterization of nerve growth factor precursor protein expression by human prostate stromal cells: a role in selective neurotrophin stimulation of prostate epithelial cell growth. *Prostate* **41:** 39–48.
40. di Sant'Agnese, P. A. and A. T. Cockett. 1996. Neuroendocrine differentiation in prostatic malignancy. *Cancer* **78:** 357–361.
41. Diaz, M., M. Abdul, and N. Hoosein. 1998. Modulation of neuroendocrine differentiation in prostate cancer by interleukin-1 and -2. *Prostate Suppl.* **8:** 32–36.
42. Downward, J., R. Riehl, L. Wu, and R. A. Weinberg. 1990. Identification of a nucleotide exchange-promoting activity for p21ras. *Proc. Natl. Acad. Sci. USA* **87:** 5998–6002.
43. Evans, C. P., F. Elfman, S. Parangi, M. Conn, G. Cunha, and M. A. Shuman. 1997. Inhibition of prostate cancer neovascularization and growth by urokinase-plasminogen activator receptor blockade. *Cancer Res.* **57:** 3594–3599.
44. Festuccia, C., F. Guerra, S. D'Ascenzo, D. Giunciuglio, A. Albini, and M. Bologna. 1998. In vitro regulation of pericellular proteolysis in prostatic tumor cells treated with bombesin. *Int. J. Cancer* **75:** 418–431.
45. Fournier, G., A. Latil, Y. Amet, J. H. Abalain, A. Volant, P. Mangin, et al. 1995. Gene amplifications in advanced-stage human prostate cancer. *Urol. Res.* **22:** 343–347.
46. Fox, S. B., R. A. Persad, N. Coleman, C. A. Day, P. B. Silcocks, and C. C. Collins. 1994. Prognostic value of c-erbB-2 and epidermal growth factor receptor in stage A1 (T1a) prostatic adenocarcinoma. *Br. J. Urol.* **74:** 214–220.
47. Frisch, S. M. and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124:** 619–626.
48. Frisch, S. M., K. Vuori, D. Kelaita, and S. Sicks. 1996. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. *J. Cell Biol.* **135:** 1377–1382.
49. Geldof, A. A., M. A. De Kleijn, B. R. Rao, and D. W. Newling. 1997. Nerve growth factor stimulates in vitro invasive capacity of DU145 human prostatic cancer cells. *J. Cancer Res. Clin. Oncol.* **123:** 107–112.
50. George, D. J., H. Suzuki, G. S. Bova, and J. T. Isaacs. 1998. Mutational analysis of the TrkA gene in prostate cancer. *Prostate* **36:** 172–180.
51. Giri, D., F. Ropiquet, and M. Ittmann. 1999. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin. Cancer Res.* **5:** 1063–1071.

52. Gleave, M., J. T. Hsieh, C. A. Gao, A. C. von Eschenbach, and L. W. Chung. 1991. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res.* **51:** 3753–3761.
53. Graham, D. K., T. L. Dawson, D. L. Mullaney, H. R. Snodgrass, and H. S. Earp. 1994. Cloning and mRNA expression analysis of a novel human protooncogene, c-mer (published erratum appears in Cell Growth Differ 1994 Sep;5(9):1022). *Cell Growth Differ.* **5:** 647–657.
54. Grasso, A. W. 1999. Neuregulin-induced signaling and cell biology in the LNCaP human prostate carcinoma cell line. Ph.D. Thesis
55. Grasso, A. W., D. Wen, C. M. Miller, J. S. Rhim, T. G. Pretlow, and H. J. Kung. 1997. ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene* **15:** 2705–2716.
56. Gresham, J., P. Margiotta, A. J. Palad, K. D. Somers, P. F. Blackmore, G. L. J. Wright, et al. 1998. Involvement of Shc in the signaling response of human prostate tumor cell lines to epidermal growth factor. *Int. J. Cancer* **77:** 923–927.
57. Gu, K., A. M. Mes-Masson, J. Gauthier, and F. Saad. 1996. Overexpression of her-2/neu in human prostate cancer and benign hyperplasia. *Cancer Lett.* **99:** 185–189.
58. Guy, P. M., J. V. Platko, L. C. Cantley, R. A. Cerione, and K. L. Carraway. 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* **91:** 8132–8136.
59. Hanks, S. K. and T. Hunter. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9:** 576–596.
60. Heinrich, P. C., I. Behrmann, G. Muller-Newen, F. Schaper, and L. Graeve. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334 (Pt 2):** 297–314.
61. Hirano, T., K. Nakajima, and M. Hibi. 1997. Signaling mechanisms through gp130: a model of the cytokine system. *Cytokine Growth Factor Rev.* **8:** 241–252.
62. Hobeika, A. C., W. Etienne, P. E. Cruz, P. S. Subramaniam, and H. M. Johnson. 1998. IFNgamma induction of p21WAF1 in prostate cancer cells: role in cell cycle, alteration of phenotype and invasive potential. *Int. J. Cancer* **77:** 138–145.
63. Hobisch, A., I. E. Eder, T. Putz, W. Horninger, G. Bartsch, H. Klocker, et al. 1998. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res.* **58:** 4640–4645.
64. Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, et al. 1992. Identification of heregulin, a specific activator of p185erbB2. *Science* **256:** 1205–1210.
65. Humphrey, P. A., X. Zhu, R. Zarnegar, P. E. Swanson, T. L. Ratliff, R. T. Vollmer, et al. 1995. Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. *Am. J. Pathol.* **147:** 386–396.
66. Iwamura, M., K. Koshiba, and A. T. Cockett. 1998. Receptors for BPH growth factors are located in some neuroendocrine cells. *Prostate Suppl.* **8:** 14–17.
67. Jarrard, D. F., B. F. Blitz, R. C. Smith, B. L. Patai, and D. B. Rukstalis. 1994. Effect of epidermal growth factor on prostate cancer cell line PC3 growth and invasion. *Prostate* **24:** 46–53.
68. Keller, E. T., C. Chang, and W. B. Ershler. 1996. Inhibition of NF-κβ activity through maintenance of IκBα levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. *J. Biol. Chem.* **271:** 26,267–26,275.
69. Khwaja, A. and J. Downward. 1997. Lack of correlation between activation of Jun-NH2-terminal kinase and induction of apoptosis after detachment of epithelial cells. *J. Cell Biol.* **139:** 1017–1023.
70. Kim, H. E., S. J. Han, T. Kasza, R. Han, H. S. Choi, K. C. Palmer, et al. 1997. Platelet-derived growth factor (PDGF)-signaling mediates radiation-induced apoptosis in human prostate cancer cells with loss of p53 function. *Int. J. Radiat. Oncol. Biol. Phys.* **39:** 731–736.

71. Kitsberg, D. I. and P. Leder. 1996. Keratinocyte growth factor induces mammary and prostatic hyperplasia and mammary adenocarcinoma in transgenic mice. *Oncogene* **13:** 2507–2515.
72. Kortylewski, M., P. C. Heinrich, A. Mackiewicz, U. Schniertshauer, U. Klingmuller, K. Nakajima, et al. 1999. Interleukin-6 and oncostatin M-induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1. *Oncogene* **18:** 3742–3753.
73. Kraus, M. H., P. Fedi, V. Starks, R. Muraro, and S. A. Aaronson. 1993. Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. *Proc. Natl. Acad. Sci. USA* **90:** 2900–2904.
74. Kuhn, E. J., R. A. Kurnot, I. A. Sesterhenn, E. H. Chang, and J. W. Moul. 1993. Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma. *J. Urol.* **150:** 1427–1433.
75. Kyriakis, J. M., H. App, X. F. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, et al. 1992. Raf-1 activates MAP kinase-kinase. *Nature* **358:** 417–421.
76. Latil, A., J. C. Baron, O. Cussenot, G. Fournier, L. Boccon-Gibod, A. Le Duc, et al. 1994. Oncogene amplifications in early-stage human prostate carcinomas. *Int. J. Cancer* **59:** 637,638.
77. Leung, H. Y., J. Weston, W. J. Gullick, and G. Williams. 1997. A potential autocrine loop between heregulin-alpha and erbB-3 receptor in human prostatic adenocarcinoma. *Br. J. Urol.* **79:** 212–216.
78. Levitzki, A. and A. Gazit. 1995. Tyrosine kinase inhibition: an approach to drug development. *Science* **267:** 1782–1788.
79. Lin, J., R. M. Adam, E. Santiestevan, and M. R. Freeman. 1999. The phosphatidylinositol 3′-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res.* **59:** 2891–2897.
80. Ling, L. and H. J. Kung. 1995. Mitogenic signals and transforming potential of Nyk, a newly identified neural cell adhesion molecule-related receptor tyrosine kinase. *Mol. Cell. Biol.* **15:** 6582–6592.
81. Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, et al. 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70:** 431–442.
82. Lyne, J. C., M. F. Melhem, G. G. Finley, D. Wen, N. Liu, D. H. Deng, et al. 1997. Tissue expression of neu differentiation factor/heregulin and its receptor complex in prostate cancer and its biologic effects on prostate cancer cells in vitro. *Cancer J. Sci. Am.* **3:** 21–30.
83. Ma, A. D., A. Metjian, S. Bagrodia, S. Taylor, and C. S. Abrams. 1998. Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. *Mol. Cell. Biol.* **18:** 4744–4751.
84. MacDonald, A. and F. K. Habib. 1992. Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *Br. J. Cancer* **65:** 177–182.
85. Manes, S., M. Llorente, R. A. Lacalle, C. Gomez-Mouton, L. Kremer, E. Mira, et al. 1999. The matrix metalloproteinase-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. *J. Biol. Chem.* **274:** 6935–6945.
86. Marchionni, M. A., A. D. Goodearl, M. S. Chen, O. Bermingham-McDonogh, C. Kirk, M. Hendricks, et al. 1993. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system (see comments). *Nature* **362:** 312–318.
87. Marengo, S. R., R. A. Sikes, P. Anezinis, S. M. Chang, and L. W. Chung. 1997. Metastasis induced by overexpression of p185neu-T after orthotopic injection into a prostatic epithelial cell line (NbE). *Mol. Carcinog.* **19:** 165–175.
88. Mark, H. F., D. Feldman, S. Das, H. Kye, S. Mark, C. L. Sun, et al. 1999. Fluorescence in situ hybridization study of HER-2/neu oncogene amplification in prostate cancer. *Exp. Mol. Pathol.* **66:** 170–178.

89. Marz, P., T. Herget, E. Lang, U. Otten, and S. Rose-John. 1997. Activation of gp130 by IL-6/soluble IL-6 receptor induces neuronal differentiation. *Eur. J. Neurosci.* **9:** 2765–2773 (published erratum appears in *Eur. J. Neurosci.* 1998 May;10(5):1936).
90. Meng, T. C. and M. F. Lin. 1998. Tyrosine phosphorylation of c-ErbB-2 is regulated by the cellular form of prostatic acid phosphatase in human prostate cancer cells. *J. Biol. Chem.* **273:** 22,096–22,104.
91. Minden, A., A. Lin, F. X. Claret, A. Abo, and M. Karin. 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81:** 1147–1157.
92. Mori, S., K. Murakami-Mori, and B. Bonavida. 1999. Interleukin-6 induces G1 arrest through induction of p27(Kip1), a cyclin-dependent kinase inhibitor, and neuron-like morphology in LNCaP prostate tumor cells. *Biochem. Biophys. Res. Commun.* **257:** 609–614.
93. Morote, J., I. De Torres, C. Caceres, C. Vallejo, S. J. Schwartz, and J. Reventos. 1999. Prognostic value of immunohistochemical expression of the c-erbB-2 oncoprotein in metastasic prostate cancer. *Int. J. Cancer* **84:** 421–425.
93a. Myers, R. B., D. Brown, D. K. Oelschlager, J. W. Waterbor, M. E. Marshall, S. Srivastava, C. R. Stockard, D. A. Urban, and W. E. Grizzle. 1996. Elevated serum levels of p105(erbB-2) in patients with advanced-stage prostate adenocarcinoma. *Int. J. Cancer* **69:** 398–402.
94. Myers, R. B., J. E. Kudlow, and W. E. Grizzle. 1993. Expression of transforming growth factor-alpha, epidermal growth factor and the epidermal growth factor receptor in adenocarcinoma of the prostate and benign prostatic hyperplasia. *Mod. Pathol.* **6:** 733–737.
95. Myers, R. B., D. Oelschlager, U. Manne, P. N. Coan, H. Weiss, and W. E. Grizzle. 1999. Androgenic regulation of growth factor and growth factor receptor expression in the CWR22 model of prostatic adenocarcinoma. *Int. J. Cancer* **82:** 424–429.
96. Myers, R. B., S. Srivastava, D. K. Oelschlager, and W. E. Grizzle. 1994. Expression of p160erbB-3 and p185erbB-2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma (see comments). *J. Natl. Cancer Inst.* **86:** 1140–1145.
97. Nagata, Y. and K. Todokoro. 1999. Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. *Blood* **94:** 853–863.
98. Nakajima, Y., A. M. DelliPizzi, C. Mallouh, and N. R. Ferreri. 1996. TNF-mediated cytotoxicity and resistance in human prostate cancer cell lines. *Prostate* **29:** 296–302.
99. Noordzij, M. A., W. M. van Weerden, C. M. A. de Ridder, T. H. van der Kwast, F. H. Schroder, and G. J. van Steenbrugge. 1996. Neuroendocrine differentiation in human prostatic tumor models. *Am. J. Pathol.* **149:** 859–871.
100. Okamoto, M., C. Lee, and R. Oyasu. 1997. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. *Cancer Res.* **57:** 141–146.
101. Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase (see comments). *Nature* **401:** 82–85.
102. Paul, A. B., E. S. Grant, and F. K. Habib. 1996. The expression and localisation of beta-nerve growth factor (beta-NGF) in benign and malignant human prostate tissue: relationship to neuroendocrine differentiation. *Br. J. Cancer* **74:** 1990–1996.
103. Peehl, D. M. and J. S. Rubin. 1995. Keratinocyte growth factor: an androgen-regulated mediator of stromal-epithelial interactions in the prostate. *World J. Urol.* **13:** 312–317.
104. Pegram, M., S. Hsu, G. Lewis, R. Pietras, M. Beryt, M. Sliwkowski, et al. 1999. Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* **18:** 2241–2251.
105. Peles, E., S. S. Bacus, R. A. Koski, H. S. Lu, D. Wen, S. G. Ogden, et al. 1992. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* **69:** 205–216.

106. Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, et al. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70:** 93–104.
107. Perry, J. E., M. E. Grossmann, and D. J. Tindall. 1998. Epidermal growth factor induces cyclin D1 in a human prostate cancer cell line. *Prostate* **35:** 117–124.
108. Pflug, B. and D. Djakiew. 1998. Expression of p75NTR in a human prostate epithelial tumor cell line reduces nerve growth factor-induced cell growth by activation of programmed cell death. *Mol. Carcinog.* **23:** 106–114.
109. Pflug, B. R., M. Onoda, J. H. Lynch, and D. Djakiew. 1992. Reduced expression of the low affinity nerve growth factor receptor in benign and malignant human prostate tissue and loss of expression in four human metastatic prostate tumor cell lines. *Cancer Res.* **52:** 5403–5406.
110. Plowman, G. D., J. M. Culouscou, G. S. Whitney, J. M. Green, G. W. Carlton, L. Foy, et al. 1993. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc. Natl. Acad. Sci. USA* **90:** 1746–1750.
111. Poller, D. N., I. Spendlove, C. Baker, R. Church, I. O. Ellis, G. D. Plowman, et al. 1992. Production and characterization of a polyclonal antibody to the c-erbB-3 protein: examination of c-erbB-3 protein expression in adenocarcinomas. *J. Pathol.* **168:** 275–280.
112. Putz, T., Z. Culig, I. E. Eder, C. Nessler-Menardi, G. Bartsch, H. Grunicke, et al. 1999. Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines. *Cancer Res.* **59:** 227–233.
113. Qiu, Y., L. Ravi, and H.-J. Kung. 1998. Requirement of ErbB2 for signaling by interleukin-6 in prostate carcinoma cells. *Nature* **393:** 83–85.
114. Qiu, Y., D. Robinson, T. G. Pretlow, and H. J. Kung. 1998. Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3′-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **95:** 3644–3649.
115. Quax, P. H., A. C. de Bart, J. A. Schalken, and J. H. Verheijen. 1997. Plasminogen activator and matrix metalloproteinase production and extracellular matrix degradation by rat prostate cancer cells in vitro: correlation with metastatic behavior in vivo. *Prostate* **32:** 196–204.
116. Rajan, R., R. Vanderslice, S. Kapur, J. Lynch, R. Thompson, and D. Djakiew. 1996. Epidermal growth factor (EGF) promotes chemomigration of a human prostate tumor cell line, and EGF immunoreactive proteins are present at sites of metastasis in the stroma of lymph nodes and medullary bone. *Prostate* **28:** 1–9.
117. Ramaswamy, S., N. Nakamura, F. Vazquez, D. B. Batt, S. Perera, T. M. Roberts, et al. 1999. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. USA* **96:** 2110–2115.
118. Robinson, D., F. He, T. Pretlow, and H. J. Kung. 1996. A tyrosine kinase profile of prostate carcinoma. *Proc. Natl. Acad. Sci. USA* **93:** 5958–5962.
119. Rohlff, C., M. V. Blagosklonny, E. Kyle, A. Kesari, I. Y. Kim, D. J. Zelner, et al. 1998. Prostate cancer cell growth inhibition by tamoxifen is associated with inhibition of protein kinase C and induction of p21(waf1/cip1). *Prostate* **37:** 51–59.
120. Romashkova, J. A. and S. S. Makarov. 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling (see comments). *Nature* **401:** 86–90.
121. Ropiquet, F., P. Berthon, J. M. Villette, G. Le Brun, N. J. Maitland, O. Cussenot, et al. 1997. Constitutive expression of FGF2/bFGF in non-tumorigenic human prostatic epithelial cells results in the acquisition of a partial neoplastic phenotype. *Int. J. Cancer* **72:** 543–547.

122. Ross, J. S., T. Nazeer, K. Church, C. Amato, H. Figge, M. D. Rifkin, et al. 1993. Contribution of HER-2/neu oncogene expression to tumor grade and DNA content analysis in the prediction of prostatic carcinoma metastasis. *Cancer* **72:** 3020–3028.
123. Ross, J. S., C. Sheehan, A. M. Hayner-Buchan, R. A. Ambros, B. V. Kallakury, R. Kaufman, et al. 1997. HER-2/neu gene amplification status in prostate cancer by fluorescence *in situ* hybridization. *Hum. Pathol.* **28:** 827–833.
124. Ross, J. S., C. E. Sheehan, A. M. Hayner-Buchan, R. A. Ambros, B. V. Kallakury, R. P. J. Kaufman, et al. 1997. Prognostic significance of HER-2/neu gene amplification status by fluorescence *in situ* hybridization of prostate carcinoma. *Cancer* **79:** 2162–2170.
125. Russell, P. J., S. Bennett, A. Joshua, Y. Yu, S. R. Downing, M. A. Hill, et al. 1999. Elevated expression of FGF-2 does not cause prostate cancer progression in LNCaP cells. *Prostate* **40:** 1–13.
126. Sadar, M. D. 1999. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. *J. Biol. Chem.* **274:** 7777–7783.
127. Saez, C., A. C. Gonzalez-Baena, M. A. Japon, J. Giraldez, D. I. Segura, J. M. Rodriguez-Vallejo, et al. 1999. Expression of basic fibroblast growth factor and its receptors FGFR1 and FGFR2 in human benign prostatic hyperplasia treated with finasteride. *Prostate* **40:** 83–88.
128. Saharinen, P., N. Ekman, K. Sarvas, P. Parker, K. Alitalo, and O. Silvennoinen. 1997. The Bmx tyrosine kinase induces activation of the Stat signaling pathway, which is specifically inhibited by protein kinase Cdelta. *Blood* **90:** 4341–4353.
129. Salim, K., M. J. Bottomley, E. Querfurth, M. J. Zvelebil, I. Gout, R. Scaife, et al. 1996. Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* **15:** 6241–6250.
130. Sanders, L. C., F. Matsumura, G. M. Bokoch, and P. de Lanerolle. 1999. Inhibition of myosin light chain kinase by p21-activated kinase (see comments). *Science* **283:** 2083–2085.
131. Saric, T. and S. A. Shain. 1998. Androgen regulation of prostate cancer cell FGF-1, FGF-2, and FGF-8: preferential down-regulation of FGF-2 transcripts. *Growth Factors* **16:** 69–87.
132. Scher, H. I., A. Sarkis, V. Reuter, D. Cohen, G. Netto, D. Petrylak, et al. 1995. Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor alpha in the progression of prostatic neoplasms. *Clin. Cancer Res.* **1:** 545–550.
133. Schoenwaelder, S. M. and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.* **11:** 274–286.
134. Schwartz, S. J., C. Caceres, J. Morote, I. De Torres, J. M. Rodriguez-Vallejo, J. Gonzalez, et al. 1999. Gains of the relative genomic content of erbB-1 and erbB-2 in prostate carcinoma and their association with metastasis. *Int. J. Oncol.* **14:** 367–371.
135. Sehgal, I., J. Bailey, K. Hitzemann, M. R. Pittelkow, and N. J. Maihle. 1994. Epidermal growth factor receptor-dependent stimulation of amphiregulin expression in androgen-stimulated human prostate cancer cells. *Mol. Biol. Cell* **5:** 339–347.
136. Sells, M. A., J. T. Boyd, and J. Chernoff. 1999. p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145:** 837–849.
137. Sells, M. A., U. G. Knaus, S. Bagrodia, D. M. Ambrose, G. M. Bokoch, and J. Chernoff. 1997. Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr. Biol.* **7:** 202–210.
138. Shen, R., T. Dorai, M. Szaboles, A. E. Katz, C. A. Olsson, and R. Buttyan. 1997. Transdifferentiation of cultured human prostate cells to a neuroendocrine cell phenotype in a hormone-depleted medium. *Urol. Res.* **3:** 67–75.
139. Siegsmund, M. J., H. Yamazaki, and I. Pastan. 1994. Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia. *J. Urol.* **151:** 1396–1399.
140. Slamon, D. J. 1998. Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER 2 overexpressiong matastatic breast cancer (HER2+/MBC) mark-

edly increases anticancer activity: a randomized, multinational controlled phrase III trial. *Progr. Proc. Am. Soc. Clin. Oncol.* **17**.

141. Sterneck, E., D. R. Kaplan, and P. F. Johnson. 1996. Interleukin-6 induces expression of peripherin and cooperates with Trk receptor signaling to promote neuronal differentiation in PC12 cells. *J. Neurochem.* **67:** 1365–1374.
142. Story, M. T., B. Livingston, L. Baeten, S. J. Swartz, S. C. Jacobs, F. P. Begun, et al. 1989. Cultured human prostate-derived fibroblasts produce a factor that stimulates their growth with properties indistinguishable from basic fibroblast growth factor. *Prostate* **15:** 355–365.
143. Sundareshan, P., R. B. Nagle, and G. T. Bowden. 1999. EGF induces the expression of matrilysin in the human prostate adenocarcinoma cell line, LNCaP (in process citation). *Prostate* **40:** 159–166.
144. Tamagnone, L., I. Lahtinen, T. Mustonen, K. Virtaneva, F. Francis, F. Muscatelli, et al. 1994. BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. *Oncogene* **9:** 3683–3688.
145. Tan, M., R. Grijalva, and D. Yu. 1999. Heregulin beta1-activated phosphatidylinositol 3-kinase enhances aggregation of MCF-7 breast cancer cells independent of extracellular signal-regulated kinase. *Cancer Res.* **59:** 1620–1625.
146. Tanaka, A., A. Furuya, M. Yamasaki, N. Hanai, K. Kuriki, T. Kamiakito, et al. 1998. High frequency of fibroblast growth factor (FGF) 8 expression in clinical prostate cancers and breast tissues, immunohistochemically demonstrated by a newly established neutralizing monoclonal antibody against FGF 8. *Cancer Res.* **58:** 2053–2056.
147. Tillotson, J. K. and D. P. Rose. 1991. Density-dependent regulation of epidermal growth factor receptor expression in DU 145 human prostate cancer cells. *Prostate* **19:** 53–61.
148. Turner, T., P. Chen, L. J. Goodly, and A. Wells. 1996. EGF receptor signaling enhances in vivo invasiveness of DU-145 human prostate carcinoma cells. *Clin. Exp. Metastasis* **14:** 409–418.
149. Turner, T., M. V. Epps-Fung, J. Kassis, and A. Wells. 1997. Molecular inhibition of phospholipase cgamma signaling abrogates DU-145 prostate tumor cell invasion. *Clin. Cancer Res.* **3:** 2275–2282.
150. Vlietstra, R. J., D. C. van Alewijk, K. G. Hermans, G. J. van Steenbrugge, and J. Trapman. 1998. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Res.* **58:** 2720–2723.
151. Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper. 1993. Mammalian *ras* interacts directly with the serine/threonine kinase Raf. *Cell* **74:** 205–214.
152. Ware, J. L. 1993. Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. *Cancer Metastasis Rev.* **12:** 287–301.
153. Watanabe, M., T. Nakada, and H. Yuta. 1999. Analysis of protooncogene c-erbB-2 in benign and malignant human prostate. *Int. Urol. Nephrol.* **31:** 61–73.
154. Wilson, C. L. and L. M. Matrisian. 1996. Matrilysin: an epithelial matrix metalloproteinase with potentially novel functions. *Int. J. Biochem. Cell Biol.* **28:** 123–136.
155. Wu, X., K. Senechal, M. S. Neshat, Y. E. Whang, and C. L. Sawyers. 1998. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. USA* **95:** 15,587–15,591.
156. Wu, Y. Y. and R. A. Bradshaw. 1996. Induction of neurite outgrowth by interleukin-6 is accompanied by activation of Stat3 signaling pathway in a variant PC12 cell (E2) line. *J. Biol. Chem.* **271:** 13,023–13,032.
157. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270:** 1326–1331.
158. Xie, H., M. A. Pallero, K. Gupta, P. Chang, M. F. Ware, W. Witke, et al. 1998. EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions inde-

pendently of the motility-associated PLCgamma signaling pathway. *J. Cell Sci.* **111 (Pt 5):** 615–624.

159. Xue, L. Y., Y. Qiu, J. He, H. J. Kung, and N. L. Oleinick. 1999. Etk/Bmx, a PH-domain containing tyrosine kinase, protects prostate cancer cells from apoptosis induced by photodynamic therapy or thapsigargin. *Oncogene* **18:** 3391–3398.
160. Yan, G., Y. Fukabori, G. McBride, S. Nikolaropolous, and W. L. McKeehan. 1993. Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. *Mol. Cell Biol.* **13:** 4513–4522.
161. Yao, R. and G. M. Cooper. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267:** 2003–2006.
162. Yeh, S., H. K. Lin, H. Y. Kang, T. H. Thin, M. F. Lin, and C. Chang. 1999. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **96:** 5458–5463.
163. Yu, D., T. Jing, B. Liu, J. Yao, M. Tan, T. J. McDonnell, et al. 1998. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. *Mol. Cell* **2:** 581–591.
164. Zetser, A., E. Gredinger, and E. Bengal. 1999. p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. *J. Biol. Chem.* **274:** 5193–5200.
165. Zhau, H. E., L. L. Pisters, M. C. Hall, L. S. Zhao, P. Troncoso, A. Pollack, et al. 1994. Biomarkers associated with prostate cancer progression. *J. Cell Biochem. Suppl.* **19:** 208–216.
166. Zhau, H. Y., J. Zhou, W. F. Symmans, B. Q. Chen, S. M. Chang, R. A. Sikes, et al. 1996. Transfected neu oncogene induces human prostate cancer metastasis. *Prostate* **28:** 73–83.
167. Zi, X., A. W. Grasso, H. J. Kung, and R. Agarwal. 1998. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res.* **58:** 1920–1929.

AQ: A MOLECULAR AND CELLULAR BIOLOGY, Dec. 2001, p. 000–000
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.24.000–000.2001

DAMD17-99-1-9021, Appendix III Vol. 21, No. 24

Neuropeptide-Induced Androgen Independence in Prostate Cancer Cells: Roles of Nonreceptor Tyrosine Kinases Etk/Bmx, Src, and Focal Adhesion Kinase

LI-FEN LEE,[1] JUNLIN GUAN,[2] YUN QIU,[3] AND HSING-JIEN KUNG*

Department of Biological Chemistry and Cancer Center, University of California at Davis, Sacramento, California 958171[1]; Cancer Biology Laboratories, Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853[2]; and Department of Laboratory Medicine and Pathology and Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455[3]
AQ: B

Received 26 March 2001/Returned for modification 22 May 2001/Accepted 6 September 2001

The bombesin/gastrin-releasing peptide (GRP) family of neuropeptides has been implicated in various in vitro and in vivo models of human malignancies including prostate cancers. It was previously shown that bombesin and/or neurotensin (NT) acts as a survival and migratory factor(s) for androgen-independent prostate cancers. However, a role in the transition from an androgen-dependent to -refractory state has not been addressed. In this study, we investigate the biological effects and signal pathways of bombesin and NT on LNCaP, a prostate cancer cell line which requires androgen for growth. We show that both neurotrophic factors can induce LNCaP growth in the absence of androgen. Concurrent transactivation of reporter genes driven by the prostate-specific antigen promoter or a promoter carrying an androgen-responsive element (ARE) indicate that growth stimulation is accompanied by androgen receptor (AR) activation. Furthermore, neurotrophic factor-induced gene activation was also present in PC3 cells transfected with the AR but not in the parental line which lacks the AR. Given that bombesin does not directly bind to the AR and is known to engage a G-protein-coupled receptor, we investigated downstream signaling events that could possibly interact with the AR pathway. We found that three nonreceptor tyrosine kinases, focal adhesion kinase (FAK), Src, and Etk/BMX play important parts in this process. Etk/Bmx activation requires FAK and Src and is critical for neurotrophic factor-induced growth, as LNCaP cells transfected with a dominant-negative Etk/BMX fail to respond to bombesin. Etk's activation requires FAK, Src, but not phosphatidylinositol 3-kinase. Likewise, bombesin-induced AR activation is inhibited by the dominant-negative mutant of either Src or FAK. Thus, in addition to defining a new G-protein pathway, this report makes the following points regarding prostate cancer. (i) Neurotrophic factors can activate the AR, thus circumventing the normal growth inhibition caused by androgen ablation. (ii) Tyrosine kinases are involved in neurotrophic factor-mediated AR activation and, as such, may serve as targets of future therapeutics, to be used in conjunction with current antihormone and antineuropeptide therapies.

AQ: C Prostate cancer is the most common noncutaneous cancer in men. The majority of patients die of disseminated disease which is hormone refractory and resistant to conventional therapies (1, 37, 57). Androgen ablation therapy, while initially effective in slowing down the progression of the disease, eventually fails, as androgen-insensitive tumors recur (20, 96).

The antiandrogen therapies usually do not eliminate the expression of the androgen receptor (AR) (87), and at least some forms of androgen insensitivity are thought to be caused by ligand-independent activation of the AR (91). For instance, Culig et al. (30) reported that the AR can be activated in the absence of androgen by growth factors such as keratinocyte growth factor (KGF), insulin-like growth factor 1 (IGF-1), and epidermal growth factor (EGF). Craft et al. (29), Yeh et al. (97) and Wen et al. (93) provide evidence that overexpression of HER2, a growth factor receptor, or its oncogenic variant Neu activates the AR in an androgen-depleted environment. Since all the receptors involved in the above-mentioned cases are tyrosine kinases, which are known initiators of phosphorylation cascades, it was postulated that direct phosphorylation of the AR may be one means to activate the receptor without androgen or to sensitize the receptor toward activation by very low levels of androgen. Indeed, direct phosphorylation of AR by serine/threonine kinases, mitogen-activated protein kinase (MAPK) (29, 97), ●●●●●●●● (AKT) (93), protein kinase A (PKA) (60, 76) and protein kinase C (PKC) (33, 45) have been reported. In most of these cases, AR activation, as measured by its ability to transcriptionally activate reporter genes, was also demonstrated. Thus, serine/threonine kinases appear to be mediators of AR activation. The type of protein kinases involved depends on the initiating growth factors and receptors. AQ: D

In addition to peptide growth factors, neuropeptides such as bombesin and neurotensin (NT) have also been implicated in prostate cancer progression. We and others previously showed that prostate cancer cells often express neuronal markers (2, 3), and some of these cells can be induced to transdifferentiate into neuroendocrine-like cells by interleukin 6 (IL-6) (66, 82), forskolin (12, 27, 28), and androgen withdrawal (19). The association of neuroendocrine cells with prostate cancers has long been recognized (2, 3, 43). Advanced prostate cancers often have increased numbers of neuroendocrine cells, and

* Corresponding author. Mailing address: UC Davis Cancer Center, Res. III, UCDMC, 4645 2nd Ave., Sacramento, CA 95817. Phone: (916) 734-1538. Fax: (916) 734-2589. E-mail: hkung@ucdavis.edu.

androgen independence is correlated with elevated levels of neuroendocrine markers in serum (2, 3, 19). Neuroendocrine cells are known to secrete neuropeptides, which are involved in diverse biological processes, including cellular proliferation, transformation, and invasion (74, 94). These neuropeptides, exemplified by bombesin and NT, have been shown to be potent in vitro mitogens (73, 74) and are implicated in a variety of human malignancies in the lung (31, 36, 89, 90), breast (61, 64), and prostate (16, 44, 51, 56). For prostate cancers, it was shown that the receptors for bombesin/gastrin-releasing peptide (GRP) are present in all prostate cancer cell lines examined, including PC3, DU145, and LNCaP (9, 13, 55), and their expression levels are increased in more-advanced tumor specimens than in less-advanced tumor specimens (55). Bombesin elicits calcium mobilization in PC3 and DU145 cells (9, 38) and enhances the invasive properties of PC3 and LNCaP cells (44). Similarly, NT induces mitogenic responses in PC3 and LNCaP cells (80). The results of these studies suggest that neuropeptides are potential prostate cancer progression factors. The mechanisms by which neuropeptides induce mitogenic and migration responses in prostate cancer cells remain unclear, although the involvement of tyrosine kinases is suggested in some of the reports (74, 75).

In this study, we report that in addition to its mitogenic and chemotactic function for prostate cancer cells, neuropeptides also activate the AR and induce androgen independence. This suggests that neuropeptides and, by extension, neuroendocrine differentiation may play a role in the transition from an androgen-dependent to -independent state. This is particularly relevant, considering that neuroendocrine differentiation of prostate cancer cells can be induced by androgen withdrawal (19) and that androgen ablation therapy is widely used in the treatment of prostate cancers. We also demonstrate in this report that three nonreceptor tyrosine kinases, focal adhesion kinase (FAK), Src, and Etk/BMX are involved in this process. All three tyrosine kinases are known to be engaged in a variety of signal pathways, including mitogenesis, migration, antiapoptosis, and reprogramming of gene expression. They have the potential to activate a number of serine/threonine kinases, which may modify the AR, leading to ligand-independent activation. These tyrosine kinases not only activate but also form a complex with one another. The receptors for bombesin and NT engage G proteins (Gαq or Gα12). Our study thus reveals a cross talk among G-proteins tyrosine kinases and nuclear receptors. In addition to providing insight into the molecular pathways whereby neuropeptides activate the AR, our results suggest that tyrosine kinase inhibitors may be useful in conjunction with androgen ablation in the treatment of prostate cancers.

MATERIALS AND METHODS

Cell culture and reagents. LNCaP cells (American Type Culture Collection, Rockville, Md.) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). PC3 cells were derived from a poorly differentiated human carcinoma and lack AR expression. PC3(AR)$_2$, a variant line which expresses AR, was established by stable transfection of a wild-type AR gene (40). The control cell line PC3(M) was developed by transfection with an empty vector carrying a hygromycin resistance gene. PC3(AR)$_2$ and PC3(M) were both maintained in 5% charcoal-stripped serum and hygromycin B (100 μg/ml). Bombesin, NT, flutamide, and Flag antibody (M2) were obtained from Sigma. EGF, phorbol myristate acetate (PMA), wortmannin, and ●●●●●●●● (PP2) were obtained from Calbiochem-Novabiochem, Ltd. Monoclonal anti-Etk was purified from Etk 13 monoclonal hybridoma culture medium using a protein A/G affinity column (Pierce). The Etk hybridoma was kindly provided by C. H. Tsai (National Taiwan University, Taipei). Anti-T7 antibody was purchased from Novagen (Madison, Wis.). Antibodies to phosphotyrosine (4G10) and to FAK and Src (monoclonal GD11 and rabbit polyclonal antibodies) were purchased from Upstate Biotechnology Inc. (UBI) (Lake Placid, N.Y.). Phospho-AKT (Ser 473) and AKT antibodies were obtained from Cell Signaling (Beverly, Mass.).

Plasmid constructs. PSA-Luc (−630/+12) was obtained by PCR-mediated amplification of human genomic DNA using oligonucleotide primers corresponding to the prostate-specific antigen (PSA) gene and ligated with *HindIII*/*XhoI*-digested PGL-3 basic vector (Promega, Madison, Wis.). ARE$_5$-luc was constructed by inserting five tandem copies of the androgen-responsive element (ARE) from the androgen-responsive, prostate-specific androgen promoter (5′-TGCAGAACAGCAAGTGCTAGC-3′) upstream of the minimal TATA box into the PGL3 basic vector (Promega). Plasmid pUXLUC (−126/−120) contains two copies of the IL-8 AP-1 binding site from the IL-8 promoter as described previously (48). The T7-tagged wild-type Etk (T7-pcDNA3Etk) or dominant-negative mutant of Etk (T7-EtkDN) as well as the dominant-negative mutant FAK (FAKY397F and FRNK) have been described previously (24, 66, 69) and c-Src (SrcKR) was kindly provided by June Zou (Cancer Center, University of California, Davis) (92)

Transfection and luciferase reporter assays. LNCaP cells were transiently transfected using Lipofectin reagent (GIBCO/BRL), and PC3(AR)$_2$ and PC3(M) cells were transiently transfected using Fugene 6 from Roche (Indianapolis, Ind.) according to the manufacturer's instructions. Briefly, luciferase reporter construct (250 ng) containing either PSA-Luc or ARE$_5$-Luc was cotransfected with 1 μg of expression vector as indicated or with pcDNA3 empty vector into LNCaP cells in six-well plates for 24 h followed by incubation in charcoal-stripped serum, phenol red-free medium with or without bombesin, NT (100 nM), or R1881 (1 nM) (methyltrienolone; DuPont New England Nuclear) as indicated for 24 h. Luciferase assays were performed on equal amounts of protein (50 μg/sample). Luciferase activities in cell lysates were measured using the Dual Luciferase assay system (Promega). *Renilla* luciferase expression plasmid, pRL-tk, was used as an internal control for transfection efficiency. The results are presented as fold induction, which is the relative luciferase activity (ratio of reporter luciferases/renilla luciferases) of the treated cells over that of the control cells.

Immunoprecipitation and Western blotting. LNCaP cells were serum starved for 24 h and then stimulated with 100 nM bombesin or NT for 30 min. Immunoprecipitation and Western blotting were performed as described previously (66). Anti-pY antibody (UBI) was used to detect tyrosine phosphorylation of FAK, Src, and Etk. Total FAK, Src, or Etk detected with anti-FAK, anti-Src, or anti-T7 antibody, respectively, was used as a loading control. Proteins were probed by primary antibody and visualized by using an ECL kit (Pierce, Rockford, Ill.) according to the manufacturer's instructions. For the dose response of Etk phosphorylation, the autoradiograms were scanned using a Gel Doc 1000 scanner (Bio-Rad), and the labeled bands were quantified using Molecular Analyst software program (Bio-Rad).

BrdU labeling proliferation assay. LNCaP-EtkWT or LNCaP-EtkDN cells (5×10^3) were seeded in 100 μl of charcoal-stripped culture medium containing serum per well in a 96-well, flat-bottom microtiter plate. The next day, the cells were treated with various amounts of bombesin (0.1 to 1,000 nM) and incubated for 4 days at 37°C with 5% CO_2. The measurement was performed according to the manufacturer's protocol for the 5-Bromo-2′-deoxyuridine Labeling and Detection Kit III (Roche). Briefly, the cells were incubated with 10 μM bromodeoxyuridine (BrdU) for 2 h at 37°C, and the labeled cells were washed with washing buffer twice and then fixed with 200 μl of precooled ethanol per well for 30 min at −20°C. After fixation, the cells were then incubated with nuclease to partially digest the DNA. The anti-BrdU-POD antibody was added to detect incorporated BrdU, and the bound antibody was visualized with the soluble chromogenic peroxidase substrate 2,2′-azinobis(3-ethylbenthiazolinesulfonic acid) (ABTS), which yielded a colorimetric reaction. The plates were measured using an enzyme-linked immunosorbent assay (ELISA) reader.

Tetrazolium compound (MTS) cell proliferation assay. The ●●●●●●●● (MTS) cell proliferation assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. LNCaP cells (5×10^3) were grown in 100 μl of charcoal-stripped culture medium containing serum per well in a 96-well, flat-bottom microtiter plate. After 24 h, the cells were treated in the absence or presence of 10 μM PP2 or flutamide for 30 min and then treated with bombesin or R1881 for another 48 to 72 h. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol.

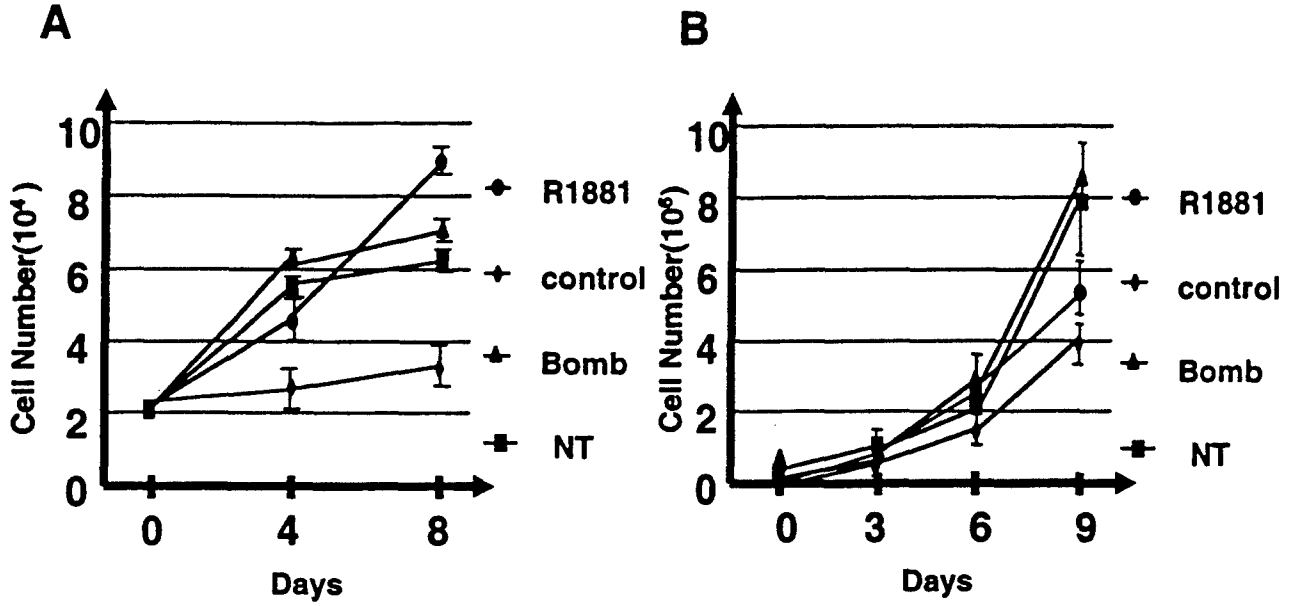

FIG. 1. Effects of bombesin (Bomb) and NT on growth of androgen-dependent prostate cancer cells. Parental LNCaP (A) and CWR22R (B) cells were plated in medium supplemented with 10% charcoal-stripped FBS with 1 nM R1881 or with 50 nM bombesin or NT, and the numbers of cells were counted at the indicated times. These results represent the averages of two independent experiments. Error bars indicate standard errors.

PSA enzyme immunoassay. The PSA protein was measured in cell culture supernatants from LNCaP cells. LNCaP cells (2×10^4) were grown in 1 ml of 2% charcoal-stripped culture medium containing serum in the presence of bombesin and NT for 72 h. PSA values were expressed in relation to cellular protein levels, which were determined by the method described by Bradford (17).

RESULTS

Bombesin and NT induce androgen-independent growth of LNCaP cells. It has been shown that bombesin and NT secreted by neuroendocrine cells exert chemotactic and mitogenic effects on tumor cells in vivo and in vitro (80, 81). These factors are also postulated to play an important role in prostate cancer progression (see introduction). To directly demonstrate their involvement in androgen-independent growth of prostate cancer cells, we measured the effects of NT and bombesin on the growth of the androgen-dependent prostate cancer cell line LNCaP (Fig. 1A). As expected, the growth rate of LNCaP was significantly reduced after androgen depletion. The addition of either NT or bombesin restores the growth of LNCaP cells, with kinetics and extent comparable to those of the synthetic androgen R1881.

We also tested another androgen-responsive prostate cancer cell line CWR22R, which was derived from a relapsed tumor (83). Although not required, androgen modulates the in vitro growth of CWR22R cells (58, 83). This was reproduced in Fig. 1B, where CWR22R cells were found to grow in the absence of androgen (control) but with an increased rate in the presence of androgen (R1881). NT and bombesin have comparable, if not higher, potencies in stimulating the growth of CWR22R cells. These experiments suggest that NT and bombesin can substitute for androgen as growth factors for androgen-responsive prostate cancer cell lines.

Bombesin and NT activate androgen-dependent promoters. The above results suggest that bombesin and NT can substitute for androgen in stimulating the growth of androgen-responsive prostate cancer cells. There are two possible mechanisms: (i) AR independent (the neurotrophic factors activate their own growth pathways without the participation of the AR) and (ii) AR dependent (the neurotrophic factors activate the AR without the participation of androgen). To distinguish between these two possibilities, we asked whether the AR is activated and whether it is required. Reporter constructs driven by the PSA promoter, known to be a target gene of the activated AR, were used to assess AR activity (78). LNCaP cells were transfected with the PSA (−630/+12) promoter-luciferase reporter plasmid and treated with R1881, NT, or bombesin in charcoal-stripped medium. At the optimal concentration of R1881 (1 nM), PSA luciferase activity was increased about 12-fold (Fig. 2A). In comparison, NT (100 nM) and bombesin (100 nM) increased PSA luciferase activity 8- and 14-fold, respectively. In support of this finding, we also observed that PSA secretion is elevated by NT and bombesin treatment, indicating that the endogenous PSA promoter was also activated (Fig. 2D).

To determine whether this activation requires the AR, we exploit the isogenic PC3 and PC3(AR)$_2$ cell lines. PC3 is a prostate cancer cell line derived from a poorly differentiated human carcinoma which lacks AR expression (34). PC3(AR)$_2$ was derived by transfection of wild-type AR gene and PC3(M) by the vector only (40). If the activation of PSA-Luc by bombesin and NT requires the AR, increased luciferase activity only in PC3(AR)$_2$, but not in PC3 or PC3(M), is expected. The results in Fig. 2B showed that PSA-Luc activity was induced 7.5-fold by R1881 and 3.5- and 4-fold by NT and bombesin, respectively, in PC3(AR)$_2$ cells but not in AR-deficient PC3 cells. These findings suggest that AR is required in the transcriptional process.

To ensure that this activation is via AR binding to the ARE as opposed to other promiscuous enhancer motifs in the PSA

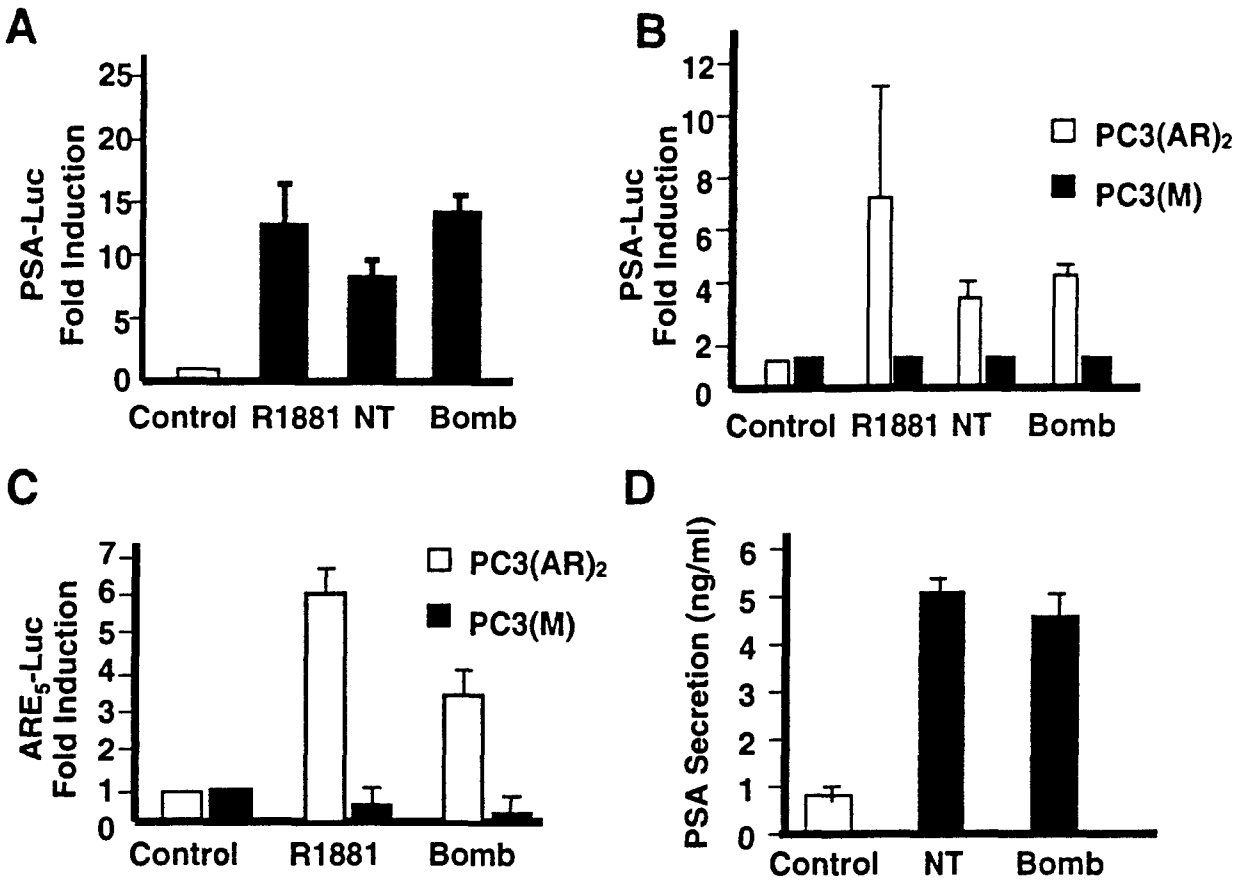

FIG. 2. Effects of bombesin (Bomb) and NT on androgen-dependent PSA transcription. Stimulation of reporter gene activity in LNCaP and PC3 cells stably expressing AR [PC3(AR)$_2$] or mock-transfected cells [PC3(M)] transfected with the reporter plasmids. (A) LNCaP cells were transiently transfected with the PSA (−630/+12 Luc) plasmid and then treated with either R1881 (1 nM), bombesin (100 nM), or NT (100 nM) for 24 h in medium with 10% charcoal-stripped FBS. (B) PC3(AR)$_2$ and PC3(M) cells were transfected with the PSA (−630/+12 Luc) plasmid. After transfection, cells were treated with either R1881(1 nM), bombesin (100 nM), or NT (100 nM) for 24 h in medium containing 5% charcoal-stripped FBS and 50 ng of hygromycin per ml. (C) PC3(AR)$_2$ and PC3(M) cells were transfected with ARE$_5$-Luc and treated with either R1881 (1 nM), bombesin (100 nM), or NT (100 nM) for 24 h in 5% charcoal-stripped FBS. The results are taken from three independent experiments. The ratio of ARE luciferase to pRL-tk luciferase represents relative luciferase activity. The fold increase indicates the ratio of the normalized luciferase activities between the cells cultured without androgen and with androgen or bombesin. (D) Regulation of PSA secretion in LNCaP cells by bombesin or NT. The cells were incubated in the presence of bombesin or NT for 72 h.

promoter, we transiently transfected PC3(AR)$_2$ and PC3(M) cells with a reporter construct, ARE$_5$-Luc that carries only ARE as the enhancer. The luciferase activity was induced 6-fold by R1881 and 3.3-fold by bombesin. These data taken together provide strong evidence that bombesin- and NT-induced responses involve AR and ARE.

Bombesin induced cell growth requires a functional AR. The induction of PSA transcriptional activity by bombesin appears to be dependent on the AR, as implied by the above experiments. To further test whether bombesin requires the AR for mitogenesis, the antiandrogen flutamide was employed. As shown in Fig. 3, preincubation of LNCaP cells with flutamide blocked bombesin-induced cell growth. Flutamide inhibited androgen-stimulated cell growth as expected. These results lend further support to the notion that bombesin-induced cell growth in LNCaP cells requires a functional AR.

F3

Tyrosine kinases activated by bombesin and NT in LNCaP. The above finding indicates that AR can be activated by neurotrophic factors. It is not intuitively obvious how neuropeptides, which do not bind nuclear receptor, but rather G-protein-coupled receptor, can function as such. The receptors for mammalian bombesin are GRP-R (gastrin-releasing peptide receptor), NMB-R (neromedullin B receptor), BRS-3 (bombesin receptor subtype 3), and the receptors for NT are NTR1 and NTR2. These receptors are coupled to Gαq and G12α (75). Gαq is known to activate PLCβ, resulting in calcium mobilization and PKC activation. We first tested whether PKC is involved in AR activation. Inhibitors of PKC did not appreciably affect AR activation by bombesin and NT (data not shown). We then turned our attention to other signal pathways. Increasing evidence suggests that G-protein signals engage tyrosine kinases including nonreceptor tyrosine kinases such as Src and Btk (32, 46, 52–54). Furthermore, as discussed earlier, phosphorylation of ARs is shown to be an alternative way of activation other than ligand binding. We then asked whether tyrosine kinases are involved in bombesin- and NT-induced AR activation. We took advantage of our knowledge of the complete tyrosine kinase expression profile of LNCaP cells as determined by an effective reverse transcription-PCR approach developed in our laboratory. Based on this approach,

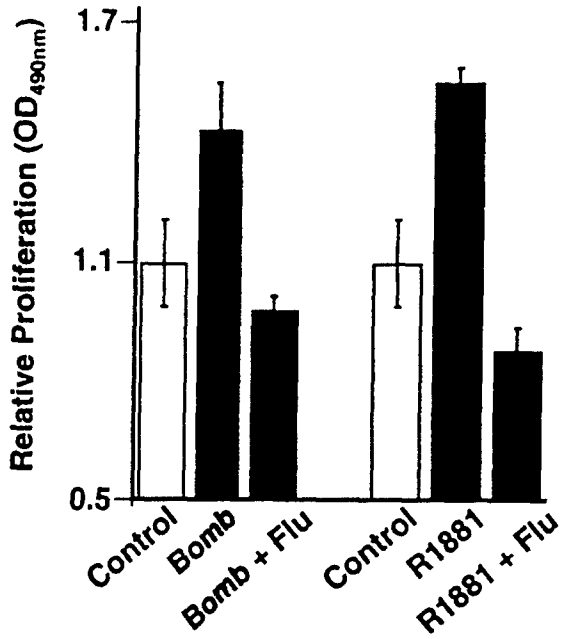

FIG. 3. Inhibitory effect of flutamide on bombesin-induced cell proliferation. LNCaP cells were preincubated in the absence or presence of flutamide (Flu) for 30 min before the addition of R1881 (1 nM) or bombesin (Bomb) (100 nM) for 72 h under charcoal-stripped serum conditions. Then 20 μl of MTS was added to each well for 2 h at 37°C. After incubation, the absorbance or optical density at a wavelength of 490 nm (OD_{490nm}) was read as described in Materials and Methods.

we know there are 21 receptor tyrosine kinases and 11 nonreceptor tyrosine kinases expressed in this cell type (50, 70). The nonreceptor tyrosine kinases are Jak, Tyk2, Src, yes, csk, FAK, Pyk2, Etk, Brk, Abl, and Arg. This information allows us to quickly screen potential tyrosine kinases activated by bombesin and NT, using immunoprecipitation with antibodies to individual kinases followed by Western blot analysis with antiphosphotyrosine antibodies. Among the tyrosine kinases screened, we found that FAK, Src, and Etk/Bmx (Fig. 4A to C, top blots) are prominently activated as reflected by the increased tyrosine phosphorylation on these proteins after neuropeptide treatments. Immunoblotting with antibodies against individual kinases confirm that similar amounts of each protein were loaded in each lane (Fig. 4A to C, bottom blots). The activation of FAK and Src is consistent with previous reports in different cell types (7, 72, 77). Our data confirm and extend these observations to the prostate LNCaP cell line. The finding regarding activation of Etk/Bmx by bombesin and NT is new but is in agreement with the reports that Gαq and Gα12 are activators of the Btk/Tec family of kinases, of which Etk/Bmx is a member (15, 46, 53).

Etk/Bmx is a tyrosine kinase carrying multiple protein-protein interaction modules including a pleckstrin homology (PH) domain, an Src homology 3 (SH3) domain, and an SH2 domain. It was first identified in bone marrow cells by Tamagnone et al. (84); it was identified independently in prostate cancer cells by our group (66, 70). In LNCaP cells, Etk is expressed at a moderate level yet plays important roles in both

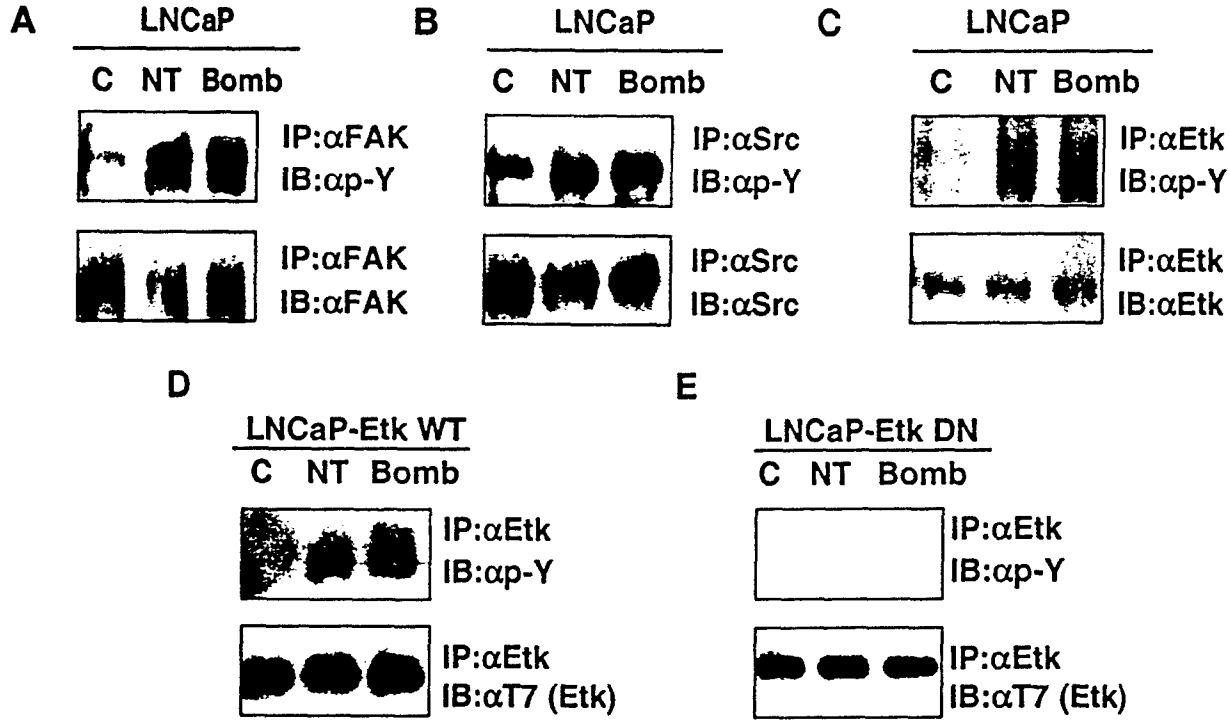

FIG. 4. Bombesin (Bomb) and NT stimulate the tyrosine phosphorylation of FAK, Src, and Etk compared to control. Antiphosphotyrosine Western blots of immunoprecipitates of FAK (A), Src (B), and Etk kinase (top blots of panels C to E). The protein expression level was confirmed by immunoblotting with antibodies to individual signaling molecules (bottom blots). (D and E) LNCaP cells were transfected with T7-Etk or T7-EtkDN (E42K and K444Q), a dominant-negative mutant, and selected by using 600 μg of G418 per ml. C, control; IP, immunoprecipitation; IB, immunoblotting; αFAK, anti-FAK antibody.

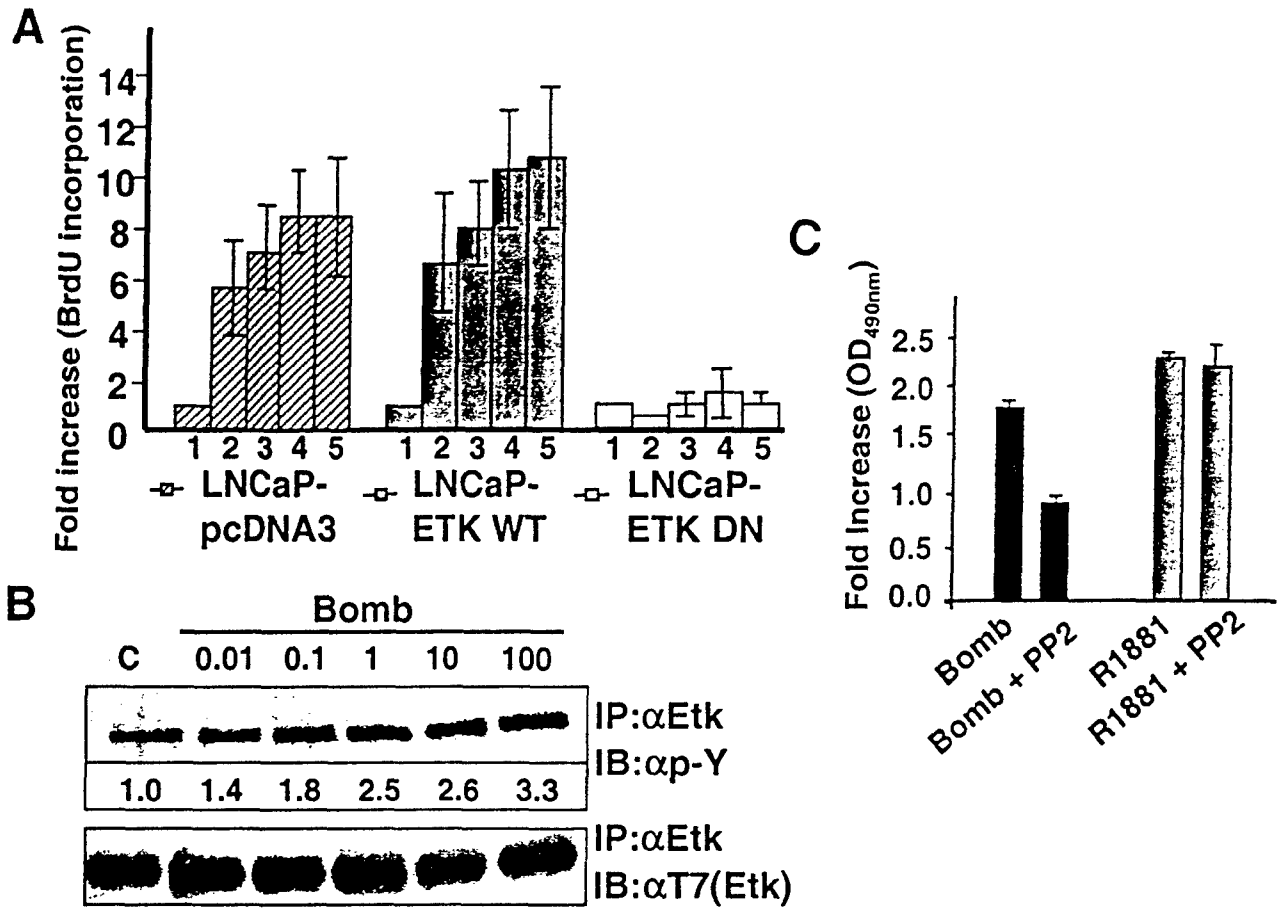

FIG. 5. Dominant-negative Etk (A) and Src (C) inhibits bombesin-mediated cell growth using the BrdU labeling proliferation assay. (A) LNCaP-pcDNA3, LNCaP-EtkWT, and LNCaP-EtkDN cells were incubated in the absence (bar 1) or presence of different concentrations of bombesin as follows: 0.1 nM (bar 2), 1 nM (bar 3), 100 nM (bar 4), and 1 μM (bar 5) in 10% charcoal-stripped FBS for 72 h. At the end of incubation, cells were fixed and stained with BrdU as specified by the manufacturer's protocol and fold increase was measured by ELISA. Each experiment was carried out in triplicate, and the error bars represent standard deviations. For each bar, the fold increase was normalized to the
AQ: S value for the control group. (B) LNCaP cells were not treated (lane C) or treated with different concentrations (nanomolar) of bombesin (Bomb) as indicated for 30 min. Tyrosine phosphorylation of Etk was analyzed by immunoprecipitation using anti-Etk antibody (IP:αEtk) followed by Western blotting (immunoblotting) with anti-pY antibody (IB:αp-Y). Anti-phospho-tyrosine (top blot) (αEtk) (active Etk) and anti-T7 (bottom
AQ: T blot) (αT7) (total Etk) antibodies were used in Western blots (immunoblots [IB]) of Etk immunoprecipitates. Numbers under the bands indicate the fold activation of Etk, as quantitated by video image densitometry. (C) LNCaP cells were preincubated in the absence or presence of PP2 (10 μM) for 30 min before the addition of R1881 (1 nM), or bombesin (Bomb) (100 nM) for 72 h under charcoal-stripped serum conditions. Then 20 μl of MTS was added to each well for 2 h at 37°C. After incubation, the absorbance or optical density at a wavelength of 490 nm (OD_{490nm}) was read.

neuroendocrine differentiation and antiapoptosis processes (66, 95). A cell line (LNCaP-EtkDN), which harbors a dominant-negative ETK KQ, shown to be effective in reversing the Etk-dependent phenotypes of LNCaP, was used in this study (66, 95). This cell line, in contrast to the parental LNCaP cell line (Fig. 4C) and a cell line transfected with wild-type Etk (Fig. 4D), failed to display neuropeptide-induced phosphorylation (Fig. 4E), confirming the kinase-dead nature of the Etk mutant and that enhanced phosphorylation is contributed primarily by autokinase activity.

Roles of Etk and Src tyrosine kinase in bombesin-mediated androgen-independent growth. The LNCaP-EtkDN cell line affords us an opportunity to assess directly the involvement of Etk in androgen-independent growth induced by bombesin. LNCaP cell lines transfected with wild-type Etk or with pcDNA vector were used as positive controls. The cell proliferation potential was determined by the measurement of ELISA-based BrdU incorporation (see Materials and Methods). As shown in Fig. 5A, in LNCaP-pcDNA3 and LNCaP-EtkWT cells, 0.1 nM bombesin induced a five- and sixfold increase in proliferation, respectively, over that of untreated samples. A 10-fold increase was observed when the concentration of bombesin was increased to 100 nM. This increase of proliferative capacity is paralleled by an increase of the Etk tyrosine phosphorylation (Fig. 5B). In stark contrast, LNCaP-EtkDN cells were not at all responsive to bombesin-stimulated growth. These data, taken together, suggest that Etk activity is critical in bombesin-induced androgen-independent cell growth. It should be noted that our previous work (95) reproduced in the present study (data not shown) showed that the growth kinetics of LNCaP-EtkDN cells is no different from that of wild-type LNCaP cells in the presence of androgen.
FS

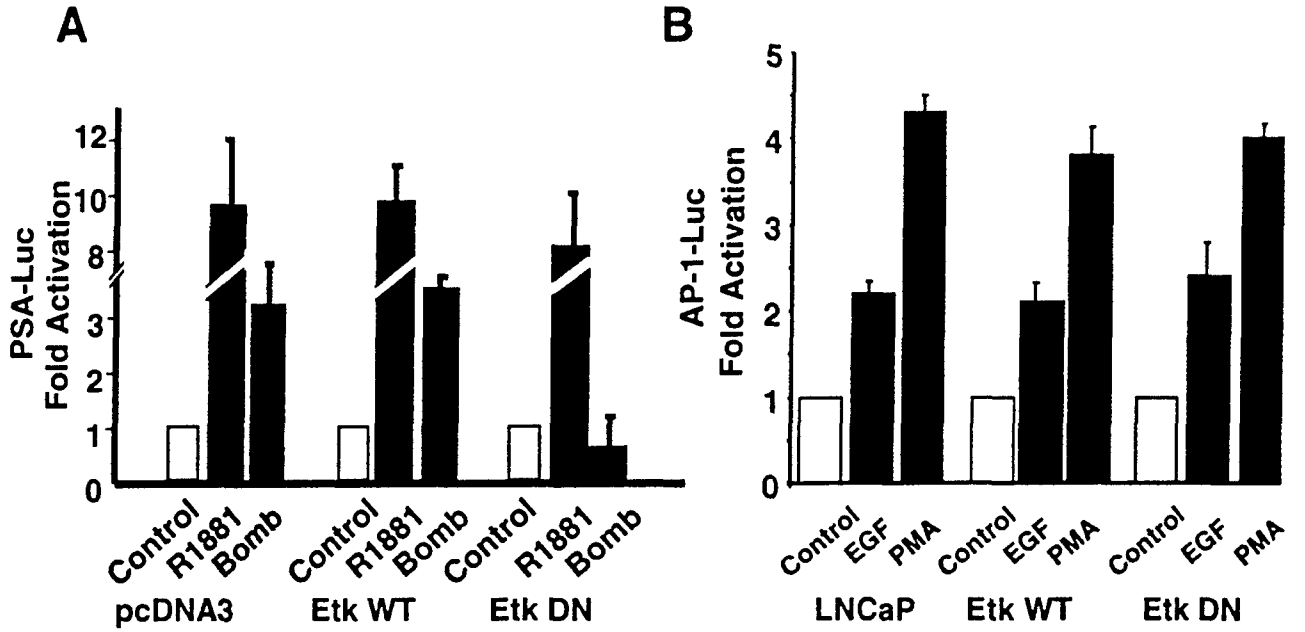

FIG. 6. Dominant-negative mutant Etk (EtkDN) blocks bombesin-induced AR pathway but not AP-1 luciferase activity. (A) LNCaP-pcDNA3, LNCaP-EtkWT, or LNCaP-EtkDN cells were cotransfected with pRL-tk vector and PSA-Luc reporter. Cells were then treated with R1881 (1 nM) or bombesin (Bomb) (100 nM) for 24 h in 5% charcoal-stripped FBS. (B) LNCaP, LNCaP-EtkWT, or LNCaP-EtkDN cells were cotransfected with pRL-tk vector and AP-1-Luc reporter (pUXLUC2X(−126/−120). Cells were then treated with EGF (10 ng/ml) or PMA (1 nM) for 24 h. The fold increase represents the ratio of the normalized luciferase activities between the cells cultured without and with EGF or PMA. The results are taken from three independent experiments.

These data suggest that Etk tyrosine kinase specifically participates in growth pathway affected by neuropeptides but not by androgen.

Taking advantage of the selective inhibitor of Src, PP2, we wish to test the involvement of Src in bombesin-induced cell proliferation of LNCaP cells. The MTS assay was performed on LNCaP cells treated with pyrazolopyrimidine PP2, at a concentration of 10 μM, which selectively inhibits Src family kinases (39, 72). As shown in Fig. 5C, PP2 specifically blocks bombesin-induced LNCaP cell proliferation, but not androgen-induced proliferation (Fig. 5C), confirming the role of Src in bombesin-induced cell proliferation.

Roles of FAK, Src, and Etk in bombesin-induced AR activation. To study whether Etk, FAK, and Src are involved in the activation of the AR by bombesin, bypassing the need for androgen, we used the ARE_5-Luc reporter transactivation assay. For AR activation by Etk, the ARE_5-Luc reporter was transfected into LNCaP-pcDNA3, LNCaP-EtkWT, or LNCaP-EtkDN cells (Fig. 6A). In LNCaP-EtkWT cells and in LNCaP-pcDNA3 cells, both R1881 and bombesin induce luciferase activity driven by the ARE, whereas in LNCaP-EtkDN cells, the activity is largely reduced in bombesin-treated cells but not in R1881-treated cells. To ensure that the observed unresponsiveness is not due to some peculiarity of LNCaP-EtkDN cells, we tested the abilities of EGF and PMA to activate AP-1 luciferase activity in this cell type (Fig. 6B); AP-1 activity is induced at the same level as those in LNCaP-EtkWT and LNCaP cells.

To test the involvement of Src and FAK in AR activation, ARE reporter construct and dominant-negative mutants of FAK or Src, FRNK and SrcKR, respectively, were cotransfected into LNCaP cells (Fig. 7). Bombesin induced ARE luciferase activity in vector-transfected cells but not in cells transfected with the dominant-negative mutants of Src and FAK. These data suggest that FAK and Src, like Etk, are also involved in bombesin-induced AR activation.

At present, we do not know exactly how this activation is accomplished, although it is unlikely that Etk directly phosphorylates AR on tyrosine residues. More likely, Etk transmits the signals through other serine/threonine kinases or coactivators, which activate the AR (see Discussion). The search for downstream signal pathways is in progress. In the ensuing section, we demonstrate data that addresses the upstream signals from bombesin to the activation of Etk.

Roles of FAK in Etk and Src activation. Having shown that Etk plays an important role in bombesin-induced androgen-independent growth and AR activation, we were interested in studying how bombesin activates Etk tyrosine kinase. The activation of Etk, like other Btk/Tec family kinases, is thought to require two steps: (i) disruption of the internal folding between the PH domain and the kinase domain by lipids, such as PIP_3 (phosphatidylinositol triphosphate) (66), or proteins (47) that have high affinity toward the PH domain; and (ii) phosphorylation of a tyrosine residue by Src-like kinase to activate the catalytic activity (6, 67). We recently reported that the FERM domain of an activated FAK associates tightly with the PH domain of Etk (25). We therefore asked whether FAK, which is activated by bombesin (Fig. 3), is involved in the activation of Etk in LNCaP cells. To this end, hemagglutinin (HA)-tagged wild-type FAK or dominant-negative mutant HA-FAKY397F or HA-FRNK was cotransfected with T7-Etk into LNCaP cells. The phosphorylation of Etk was measured after

F8 bombesin treatment. Figure 8 shows that bombesin strongly activates Etk in vector-transfected LNCaP (Fig. 8A, lanes 1 and 2) or in wild-type FAK-transfected LNCaP (Fig. 8A, lanes 4 and 5). FAKY397F has a greatly diminished kinase activity and failed to bind both Src and PI3K (21). Another dominant-negative mutant, FRNK, is a C-terminal variant of FAK, which does not have a kinase domain but retains the focal contact domain (69). Expression of either dominant-negative mutant of FAK (HA-FAKY397F or HA-FRNK) greatly reduced Etk activation by bombesin (Fig. 8A, lanes 6 and 7), suggesting FAK is an activator of Etk. At the same time, HA-FAKY397F also diminishes the activity of Src (Fig. 8B), suggesting that FAK Y397 binding contributes to Src activation. There are several mechanisms by which FAK can activate Etk. FAK can activate Etk directly or indirectly through Src and PI3K. The above mechanisms are not mutually exclusive. We proceeded to investigate the involvement of Src and PI3K in Etk activation.

Role of Src in Etk activation. To test the role of Src in Etk activation, we used PP2 or dominant-negative mutant SrcKR. Both experiments yielded convergent results, indicating that Src activity is required for bombesin-induced Etk activation.
F9 Figure 9A showed that PP2 treatment or SrcKR transfection significantly diminishes Etk activity (Fig. 9A, compare lanes 4 and 6 to lane 2).

Role of PI3K in Etk activation. We previously showed that Etk, like other Btk/Tec family kinases, is activated by PI3K, presumably via the binding of the Etk PH domain to PIP3, the metabolic product of PI3K. Specifically, we showed that PI3K is required for IL-6-induced activation of Etk (66). Since FAK is known to be an activator of PI3K, we were curious whether PI3K is involved in Etk activation. In contrast to PP2, wortmannin, an inhibitor for PI3K, had little effect on bombesin-induced activation (Fig. 9A, lane 3 versus lane 2). To ensure that wortmannin worked as intended, we included AKT phosphorylation (a known indicator of PI3K activity) as a control. Wortmannin completely abolishes the phosphorylation of AKT, based on the lack of signal in the immunoblot with phospho-AKT antibody (Fig. 9B, lane 3)

To further demonstrate that FAK activation of Etk is through Src, but not PI3K, we used a FAK mutant, FAKD395A, that preserves the Y397 binding context for Src but not PI3K (68). We predicted that this mutant should still activate Etk, and this is indeed the case: FAKD395A only slightly decreased Etk activation (Fig. 8, compare lanes 2 and 3) confirming a major role for Src but not PI3K in Etk activation.

FAK, Src, and Etk form a stable in vivo complex. Our data provide evidence that the three nonreceptor kinases FAK, Src, and Etk are all activated upon bombesin treatment of LNCaP cells. Since we know from previous reports for different cell types that FAK interacts with Src (21), Src interacts with Etk (85), and Etk interacts with FAK (25), we were curious to determine whether a complex involving all three components can be found in bombesin-treated LNCaP cells. The results using the LNCaP-EtkWT cells showed that immunoprecipitates of FAK contain Etk, based on Western blot results with
F10 T7 (Etk) antibody (Fig. 10C). The formation of this complex, however, does not depend on bombesin treatment, indicating that in LNCaP cells, the Etk and FAK complex has already formed (Fig. 10C, lanes 1 and 2). By contrast, the formation of

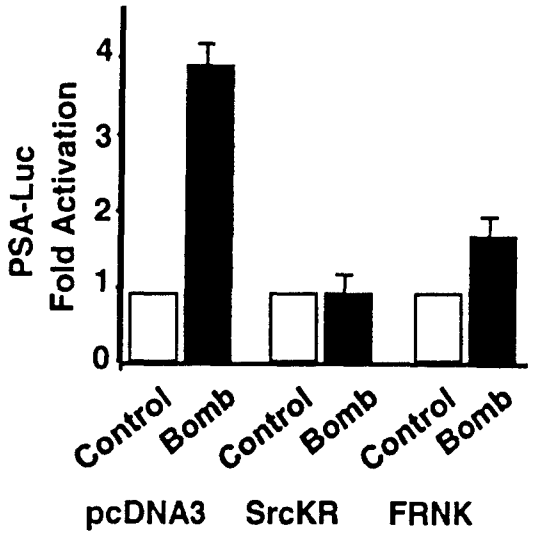

FIG. 7. Dominant-negative mutant of Src (SrcKR) and FAK (FRNK) blocks bombesin-induced AR pathway. LNCaP cells were cotransfected with the PSA-Luc reporter and a pRL-tk reporter plus SrcKR or FRNK or an empty vector and cultured in 5% charcoal-stripped FBS. The ratio of ARE luciferase to pRL-tk luciferase represents relative luciferase activity. The fold increase indicates the ratio of the normalized luciferase activities between the cells cultured without bombesin and with bombesin (Bomb). The results are taken from three independent experiments.

a complex between Src and Etk depends on bombesin (Fig. 10G, lanes 1 and 2). To further confirm that these associations are specific, we also performed experiments using a nonspecific Flag antibody. The results showed that immunoprecipitates of FAK and Src were not detected after immunoblotting with Flag antibody (Fig. 10D and H), which suggests that the interactions of FAK-Etk and Src-Etk are specific. These findings, together with those of Salazar and Rozengut (77) showing that FAK-Src association depends on treatment with bombesin, suggest that FAK is likely the scaffold that pulls these two components together. Based on the above results, we showed that bombesin induces the formation of a signal complex with three activated tyrosine kinases that has the potential to transmit a phosphorylation cascade that can modify the AR. It is likely the combined action of these phosphorylations that make this pathway particularly active.

DISCUSSION

There are several significant findings in this study. (i) We establish that neuropeptides such as bombesin and NT are able to substitute for androgen in sustaining the growth of androgen-dependent LNCaP cells, raising the possibility that neuroendocrine cells and their released paracrine factors play important roles in prostate cancer progression. (ii) We show that these neuropeptides are able to activate androgen-dependent promoters and that this process requires a functional AR, implicating their involvement during the transition from an androgen-dependent to -independent state. (iii) We show that bombesin and NT activate a signal complex involving three nonreceptor tyrosine kinases, FAK, Src, and Etk/Bmx, connecting G-protein signaling to that of tyrosine kinases in prostate cancer cells. These kinases are known to be involved in cell

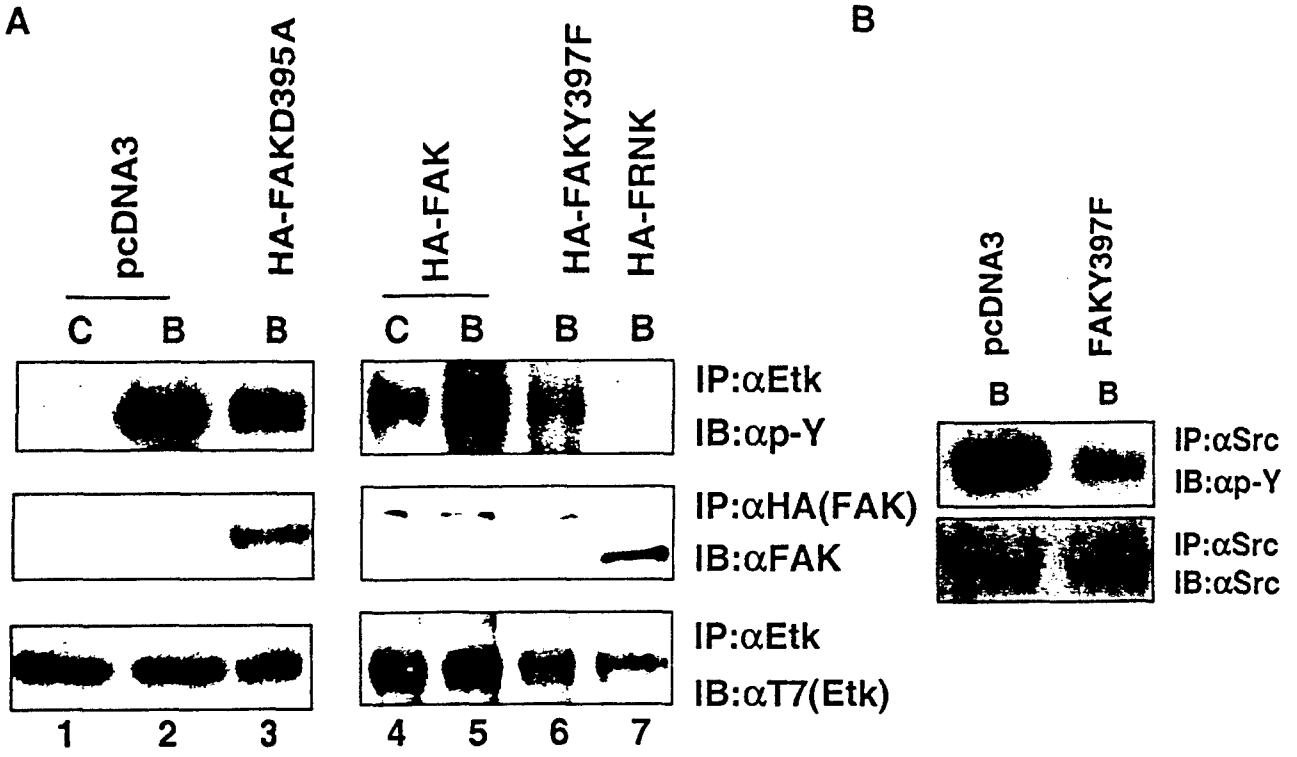

FIG. 8. Dominant-negative mutants of FAK, HA-FAKY397F, and HA-FRNK, but not HA-FAKD395A, blocked Etk activation in response to bombesin. (A) Cells were cotransfected with wild-type Etk or T7-Etk with one of the following plasmids: vector, wild-type FAK, HA-FAK, or dominant-negative mutant HA-FAKD395A, HA-FAKY397F, or HA-FRNK. After transfection, LNCaP cells were then treated with 100 nM bombesin (lanes B) or untreated control (lanes C) for 30 min as indicated and subsequently lysed. Tyrosine phosphorylation of Etk was analyzed by immunoprecipitation using anti-Etk antibody (IP:αEtk) followed by Western blotting (immunoblotting) with anti-pY antibody (IB:αp-Y) 4G10 (top blots). The membrane was analyzed further by Western blotting using T7 antibody [IB:αT7(Etk)] (bottom blots). Half of the cell lysates described for Etk were immunoprecipitated with HA antibody [IP:αHA(FAK)] followed by blotting with FAK polyclonal antibody (IB:αFAK) (middle blots). (B) LNCaP cells were transfected with FAKY397F or empty vector, pcDNA3, and the lysates were immunoprecipitated with monoclonal Src antibody (IP:αSrc) and immunoblotted with anti-pY antibody (IB:αp-Y) (top blot) and anti-Src polyclonal antibody (IB:αSrc) (bottom blot).

motility, transformation, and antiapoptosis, respectively, which may account for some of the properties associated with neuropeptides. (iv) We present evidence that these tyrosine kinases are also involved in the induction of androgen independence, identifying them as potential therapeutic targets. (v) Finally, we demonstrate that Etk/Bmx, can be activated by FAK, possibly through Src, without significant involvement of PI3K, providing new insight into the activation mechanism of Btk/Tec family kinases.

Neuroendocrine cells and prostate cancer progression. It has long been recognized that neuroendocrine cells are present and intermingled with healthy prostate or prostate cancer epithelial cells (2, 3). Some reports suggest that neuroendocrine cells increase in number during prostate cancer progression (4, 5). It has been proposed that cells may act as a source for paracrine factors that support androgen-independent growth, survival, and migration of the surrounding cancer cells (8, 10). We and others showed that LNCaP can be transdifferentiated by cytokine IL-6 (66) or cyclic AMP agonists (27, 28) into neuroendocrine cells with neuronal morphology. These cells are postmitotic and unable to grow but release neurotrophic factors which potentially can stimulate the growth of the surrounding undiffentiated cancer cells. Among the factors released by neuroendocrine cells, bombesin/GRP has been studied most extensively as an autocrine and paracrine growth factor for many tumor types (9, 42). Bombesin is both a growth factor and migration factor for fibroblasts, lung cancer cells, and prostate cancer cells. In this study, we showed that it may also be a progression factor for androgen independence. Therapeutic modalities based on antagonists of GRP or its receptor have already been developed, and some are currently undergoing clinical trials (79). Our results that bombesin/GRP induces androgen independence via tyrosine kinases suggest that tyrosine kinase inhibitors, which have shown great promise in cancer treatments, may also be used as a combination therapy.

Signal pathways activated by neuropeptides. Both bombesin and NT bind to the G-protein-coupled receptor (14, 98). The engagement of Gαq to the receptor liberates Gβγ, which activates phospholipase β (PLCβ) (41, 62). PLCβ produces inositol 1,4,5-triphosphate which mobilizes Ca^{2+} from internal stores and diacylglycerol which in turn, activates PKC (23, 35, 59). A recent study by Buhl et al. showed that Gα12 is also activated by bombesin (18). Gα12 directly associates with RhoGEF which activates small G-protein Rho (27, 28). Activation of Rho leads to actin polymerization, an important step in cell motility. How and whether these signals generated from G

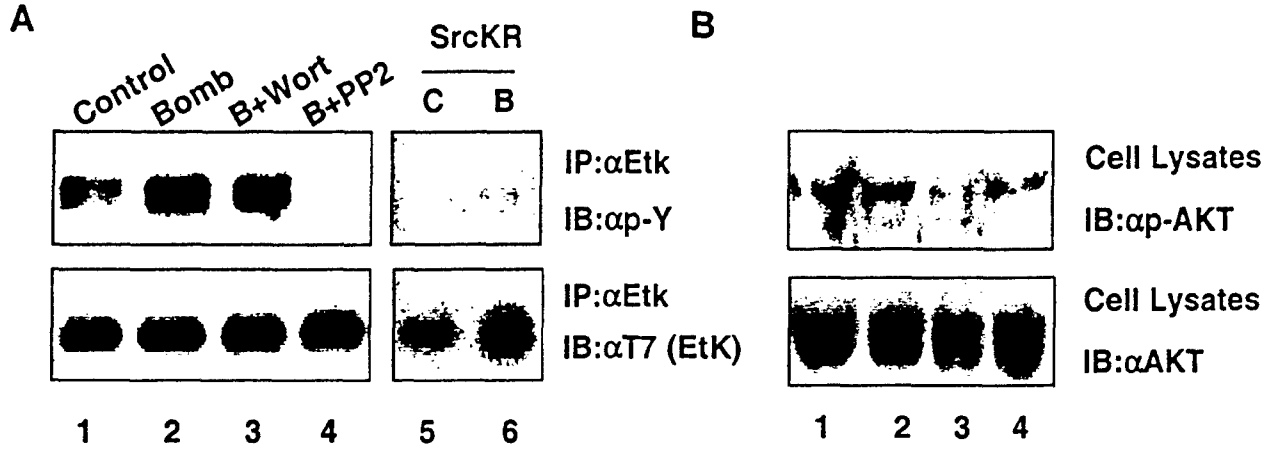

FIG. 9. Src, but not PI3K, is critical in bombesin-induced Etk activation. (A and B) PP2 blocks the activation of Etk by bombesin (Bomb). LNCaP-EtkWT cells were serum starved for 24 h. The cells were then pretreated with 100 nM wortmannin (Wort), 10 μM PP2, or dimethyl sulfoxide (control or C [lanes 1 and 5]) for 30 min and then treated with bombesin for 30 min as indicated (Bomb or B) (lanes 2 to 4). A dominant-negative Src (c-SrcKR) blocks the activation of Etk by bombesin (lanes 5 and 6). LNCaP cells were cotransfected with Src and Etk dominant-negative mutant (EtkDN and c-SrcKR). At 24 h posttransfection, cells were serum starved for 24 h and treated with bombesin (lane B) for 30 min as indicated. The cell extracts were immunoprecipitated with anti-Etk (IP:αEtk) and then immunoblotted with anti-pY antibody (IB:αp-Y) (top blots) and anti-T7 antibody [IB:αT7 (EtK)] (bottom blots). (B) The cell extracts were immunoblotted with anti-phospho-AKT antibody (IB:αp-AKT) (top blot) and anti-AKT antibody (IB:αAKT) (bottom blot).

proteins are connected to AR activation is presently unclear. Salazar and Rozengurt (77) showed that in fibroblasts, bombesin-induced actin clustering leads to the activation of FAK tyrosine kinase and a rapid increase in the formation of FAK-Src complexes. This process depends on the integrity of the actin filament network, but not on Ca^{2+} or PI3K (75). Our results for LNCaP cells are in agreement with this finding, and F11 we propose the following model (Fig. 11) depicting the signals connecting bombesin to the activation of three kinases and eventually to the AR.

Involvement of nonreceptor tyrosine kinases FAK, Src, and Etk/Bmx. FAK was originally discovered as a substrate of Src and a kinase activated by integrin clustering (63). FAK, localized in focal adhesion and membrane ruffles, is now thought to play a role in cell motility rather than the formation of focal AQ: M complexes. FAK is comprised of three domains, the N-terminal FERM domain, a protein-protein interaction domain with homology to band 4.1, the tyrosine kinase domain, and the C-terminal F-actin binding domain. Initial activation of the FAK autokinase is accomplished by actin polymerization or integrin clustering (71), leading to the phosphorylation of Y397, which then serves as the anchor site for either Src or PI3K. The interaction of Y397 with the SH2 domain of Src activates Src kinase activity. The activated Src in turn phosphorylates Y576 and Y577 of FAK, leading to the full activation of FAK (72, 74, 75, 77). This is a case where two kinases act synergistically with each other to reach maximal activity. One of the dominant-negative mutants used in this study, Y397F, is not able to bind Src, and as a result achieves only basal activation levels. The second mutant, FRNK, corresponds to the C-terminal part of FAK which competes with wild-type FAK for localization to focal contacts but lacks kinase activity to transmit a downstream signal. FRNK was shown to be a potent dominant-negative mutant in blocking migration and other phenotypes induced by FAK (69). A third mutant, D395A, was engineered to maintain the binding context around Y397 for Src, but not PI3K. As a result, this mutant is capable of activating only Src, not PI3K (24).

In addition to FAK and Src, our survey of bombesin-activated tyrosine kinases revealed that Etk/Bmx is also activated. Etk/BMX is a tyrosine kinase characterized by having a PH domain at the N terminus and is in the Btk or Tec family of kinases (84, 86, 88). The PH domain, which has a protein-lipid interaction domain as well as a protein-protein interaction domain, regulates Etk activity by a two-step mechanism. The first involves the binding of the PH domain to its lipid-ligand PI(3,4,5)P3, a product of PI3K, or a protein-ligand such as AQ: N protein-tyrosine phosphatase D1 (47). This binding presumably opens up the kinase domain, allowing Src-like kinases to phosphorylate tyrosine residue 566, which activates the intrinsic kinase activity of Etk (26, 49). Recently, we showed that the FERM domain of FAK associates with Etk and serves as a ligand to activate Etk (25). The present study extends this observation and demonstrates that FAK, Src, and Etk are engaged in the formation of a complex in bombesin-treated cells. The association between FAK and Etk appears to be preformed, whereas that of Src is induced by neurotrophic factors (Fig. 11). Dominant-negative mutants of FAK and Src inhibitors abolish the Etk activation, while the PI3K inhibitor wortmannin has no effect. This is consistent with the model depicted in Fig. 11 that FAK, Src, and Etk form a complex, with the potential for mutual activations. Within this complex, FAK activates Src (77) and Src activates Etk (85). At the same time, Src is known to activate FAK (21) and FAK has been shown to activate Etk (25). The result is the activation of three tyrosine kinases which each have the potential to transmit phosphorylation signals to the nucleus, resulting in AR activation. Although in this study we focused on the androgen-independent growth aspect of LNCaP cells, it is likely that this complex is also involved in enhanced migration (●. ●. Yang and ●. Evans, personal communication). AQ: O

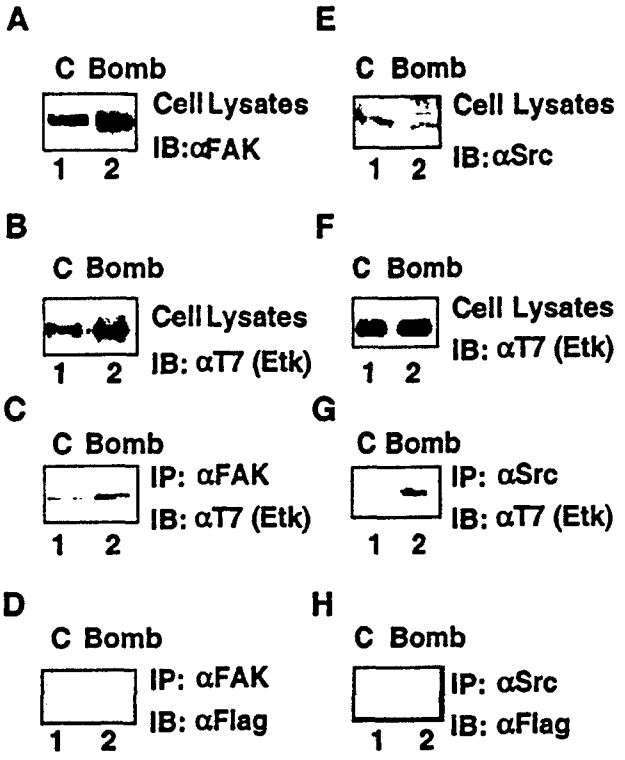

FIG. 10. Etk interacts with FAK and Src in cells. LNCaP-EtkWT cells were either not treated or treated with 100 nM bombesin for 30 min. After 48 h posttransfection, cells were not treated (control [C]) (lanes 1) or treated with 100 nM bombesin (Bomb) (lanes 2). The expression of FAK, Etk, or Src was analyzed by Western blotting (immunoblotting [IB]) using either anti-FAK (αFAK) (A), anti-T7 (Etk) [αT7 (Etk)] (B and F), or anti-Src (αSrc) (E) antibodies, respectively. Half of the cell lysates used above for panels A and D were incubated with anti-FAK antibody and anti-Src antibody, and the immunoprecipitates were then Western blotted (immunoblotted) with anti-T7 (Etk) antibody [IB: αT7 (Etk)] (C and G), respectively, to detect the association of Etk with FAK and Src. The blots from panels C and G were stripped and then Western blotted with anti-Flag antibody (IB: αFLAG) (D and H).

Role of Etk in androgen independence. Previous work from this and other labs showed that Etk/Bmx is involved in cellular transformation, antiapoptosis, and differentiation processes of various cell types (66, 85, 95). The present study showed that Etk and Src are required for androgen-independent growth and the activation of the AR pathway. We have not addressed how Etk/Bmx or the FAK-Src-Etk complex channels the signals to the AR. We presume that it is through phosphorylation of the AR by downstream kinases. As discussed earlier, MAPK and AKT have been found to be mediators of androgen-independent activation of the AR. Both kinase pathways are activated by FAK and Src, and AKT was shown to be activated by at least one member of the Tec family of kinases (22). Indeed, recently we have observed that MAPK can be activated by bombesin and that activated MAPK phosphorylated the AR in an in vitro kinase assay (L.-F. Lee and H.-J. Kung, unpublished data). In addition, Etk has the potential to activate a number of other serine kinases, such as PAK (11) and PKC (65), which may also participate in a yet unknown role in AR activation. Alternatively, the target of phosphorylation may be coactivators or corepressors of AR. The association of coactivators or dissociation of corepressors are responsible for AR activation. Phosphorylation of these modulators may change their affinities towards AR, resulting in activation of the AR. Among the known targets of Etk, STAT3, when phosphorylated and activated, was shown to render AR active (26, 85). Experiments to define the downstream pathway leading to AR activation by Etk or the FAK-Src-Etk complex are in progress.

AQ: P

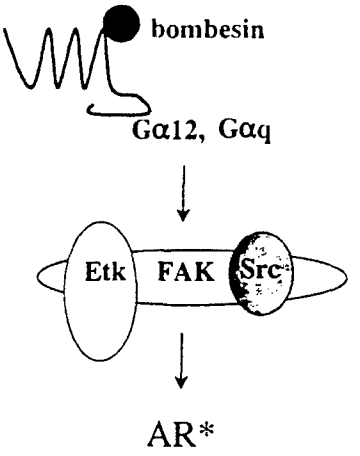

FIG. 11. Summary of the signaling pathway that connects neuropeptides to the AR.

In summary, neurotrophic factors have been implicated in prostate cancer progression (9). Here we show that, in addition to their roles in the stimulation of growth and metastasis of prostate cancer cells, they also induce androgen independence and thus may be involved in the initial transition from an androgen-dependent to -independent state. They do so by activating a signal complex consisting of three nonreceptor tyrosine kinases, FAK, Src, and Etk. This new pathway integrates signals generated by G-protein-coupled receptors, tyrosine kinases, and hormone receptor.

ACKNOWLEDGMENTS

We thank Chris Evan, who kindly provided the PC3(AR)$_2$ and PC3(M) cells, and David L. Boucher for critical reading of the manuscript.

This study was supported in part by a United States Department of Defense postdoctoral fellowship (PC001247 to L.-F.L.) and by grants from the California Prostate Research program, Department of Defense (PC970090), and the National Institutes of Health (CA39207 and CA57179 to H.-J.K.)

REFERENCES

1. **Abi-Aad, A. S., and R. J. Opsomer.** 1996. Prostate cancer-treatment of disseminated disease. Acta Urol. Belg. **64**:67–76.
2. **Abrahamsson, P. A.** 1999. Neuroendocrine cells in tumour growth of the prostate. Endocr. Relat. Cancer **6**:503–519.
3. **Abrahamsson, P. A.** 1999. Neuroendocrine differentiation in prostatic carcinoma. Prostate **39**:135–148.
4. **Abrahamsson, P. A., S. Falkmer, K. Falt, and L. Grimelius.** 1989. The course of neuroendocrine differentiation in prostatic carcinomas. An immunohistochemical study testing chromogranin A as an "endocrine marker." Pathol. Res. Pract. **185**:373–380.
5. **Abrahamsson, P. A., L. B. Wadstrom, J. Alumets, S. Falkmer, and L. Grimelius.** 1987. Peptide-hormone- and serotonin-immunoreactive tumour cells in carcinoma of the prostate. Pathol. Res. Pract. **182**:298–307.
6. **Afar, D. E., H. Park, B. W. Howell, D. J. Rawlings, J. Cooper, and O. N. Witte.** 1996. Regulation of Btk by Src family tyrosine kinases. Mol. Cell. Biol. **16**:3465–3471.

7. **Allard, P., P. Beaulieu, A. Aprikian, and S. Chevalier.** 2000. Bombesin modulates the association of Src with a nuclear 110-kd protein expressed in dividing prostate cells. J. Androl. **21**:367–375.
8. **Aprikian, A. G., C. Cordon-Cardo, W. R. Fair, Z. F. Zhang, M. Bazinet, S. M. Hamdy, and V. E. Reuter.** 1994. Neuroendocrine differentiation in metastatic prostatic adenocarcinoma. J. Urol. **151**:914–919.
9. **Aprikian, A. G., K. Han, S. Chevalier, M. Bazinet, and J. Viallet.** 1996. Bombesin specifically induces intracellular calcium mobilization via gastrin-releasing peptide receptors in human prostate cancer cells. J. Mol. Endocrinol. **16**:297–306.
10. **Aprikian, A. G., K. Han, L. Guy, F. Landry, L. R. Begin, and S. Chevalier.** 1998. Neuroendocrine differentiation and the bombesin/gastrin-releasing peptide family of neuropeptides in the progression of human prostate cancer. Prostate Suppl. **8**:52–61.
11. **Bagheri-Yarmand, R., M. Mandal, A. H. Taludker, R. A. Wang, R. K. Vadlamudi, H. J. Kung, and R. Kumar.** 2001. Etk/Bmx tyrosine kinase activates PAK-1 and regulates the tumorigenicity of breast cancer cells. J. Biol. Chem. **276**:29403–29409.
12. **Bang, Y. J., F. Pirnia, W. G. Fang, W. K. Kang, O. Sartor, L. Whitesell, M. J. Ha, M. Tsokos, M. D. Sheahan, and P. Nguyen.** 1994. Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. Proc. Natl. Acad. Sci. USA **91**:5330–5334.
13. **Bartholdi, M. F., J. M. Wu, H. Pu, P. Troncoso, P. A. Eden, and R. I. Feldman.** 1998. In situ hybridization for gastrin-releasing peptide receptor (GRP receptor) expression in prostatic carcinoma. Int. J. Cancer **79**:82–90.
14. **Battey, J. F., J. M. Way, M. H. Corjay, H. Shapira, K. Kusano, R. Harkins, J. M. Wu, T. Slattery, E. Mann, and R. I. Feldman.** 1991. Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. Proc. Natl. Acad. Sci. USA **88**:395–399.
15. **Bence, K., W. Ma, T. Kozasa, and X. Y. Huang.** 1997. Direct stimulation of Bruton's tyrosine kinase by G(q)-protein alpha-subunit. Nature **389**:296–299.
16. **Bologna, M., C. Festuccia, P. Muzi, L. Biordi, and M. Ciomei.** 1989. Bombesin stimulates growth of human prostatic cancer cells in vitro. Cancer **63**:1714–1720.
17. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
18. **Buhl, A. M., N. L. Johnson, N. Dhanasekaran, and G. L. Johnson.** 1995. G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J. Biol. Chem. **270**:24631–24634.
19. **Burchardt, T., M. Burchardt, M. W. Chen, Y. Cao, A. de la Taille, A. Shabsigh, O. Hayek, T. Dorai, and R. Buttyan.** 1999. Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype in vitro and in vivo. J. Urol. **162**:1800–1805.
20. **Carter, H. B., and J. T. Isaacs.** 1990. Overview of hormonal therapy for prostate cancer. Prog. Clin. Biol. Res. **350**:129–140.
21. **Cary, L. A., and J. L. Guan.** 1999. Focal adhesion kinase in integrin-mediated signaling. Front. Biosci. **4**:D102–D113.
22. **Chan, T. O., S. E. Rittenhouse, and P. N. Tsichlis.** 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu. Rev. Biochem. **68**:965–1014.
23. **Charlesworth, A., S. Broad, and E. Rozengurt.** 1996. The bombesin/GRP receptor transfected into Rat-1 fibroblasts couples to phospholipase C activation, tyrosine phosphorylation of p125FAK and paxillin and cell proliferation. Oncogene **12**:1337–1345.
24. **Chen, H. C., P. A. Appeddu, H. Isoda, and J. L. Guan.** 1996. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J. Biol. Chem. **271**:26329–26334.
25. **Chen, R., O. Kim, M. Li, X. Xiong, J. L. Guan, H. J. Kung, H. Chen, Y. Shimizu, and Y. Qiu.** 2001. Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain. Nat. Cell Biol. **3**:439–444.
26. **Chen, T., L. H. Wang, and W. L. Farrar.** 2000. Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. Cancer Res. **60**:2132–2135.
27. **Cox, M. E., P. D. Deeble, E. A. Bissonette, and S. J. Parsons.** 2000. Activated 3′,5′-cyclic AMP-dependent protein kinase is sufficient to induce neuroendocrine-like differentiation of the LNCaP prostate tumor cell line. J. Biol. Chem. **275**:13812–13818.
28. **Cox, M. E., P. D. Deeble, S. Lakhani, and S. J. Parsons.** 1999. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. Cancer Res. **59**:3821–3830.
29. **Craft, N., Y. Shostak, M. Carey, and C. L. Sawyers.** 1999. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat. Med. **5**:280–285.
30. **Culig, Z., A. Hobisch, M. V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, G. Bartsch, and H. Klocker.** 1994. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res. **54**:5474–5478.
31. **Cuttitta, F., D. N. Carney, J. Mulshine, T. W. Moody, J. Fedorko, A. Fischler, and J. D. Minna.** 1985. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature **316**:823–826.
32. **Della, R. G., T. van Biesen, Y. Daaka, D. K. Luttrell, L. M. Luttrell, and R. J. Lefkowitz.** 1997. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. J. Biol. Chem. **272**:19125–19132.
33. **de Ruiter, P. E., R. Teuwen, J. Trapman, R. Dijkema, and A. O. Brinkmann.** 1995. Synergism between androgens and protein kinase-C on androgen-regulated gene expression. Mol. Cell Endocrinol. **110**:R1–R6.
34. **Edelstein, R. A., M. C. Carr, R. Caesar, M. Young, A. Atala, and M. R. Freeman.** 1994. Detection of human androgen receptor mRNA expression abnormalities by competitive PCR. DNA Cell Biol. **13**:265–273.
35. **Erusalimsky, J. D., I. Friedberg, and E. Rozengurt.** 1988. Bombesin, diacylglycerols, and phorbol esters rapidly stimulate the phosphorylation of an Mr = 80,000 protein kinase C substrate in permeabilized 3T3 cells. Effect of guanine nucleotides. J. Biol. Chem. **263**:19188–19194.
36. **Frankel, A., M. S. Tsao, and J. Viallet.** 1994. Receptor subtype expression and responsiveness to bombesin in cultured human bronchial epithelial cells. Cancer Res. **54**:1613–1616.
37. **Gonzalez, M. M., V. F. Gomez, and C. L. Alvarez.** 1997. Disseminated carcinoma of the prostate: monotherapy or complete androgenic blockade? Arch. Esp. Urol. **50**:1067–1076. AQ: Q
38. **Han, K., J. Viallet, S. Chevalier, W. Zheng, M. Bazinet, and A. G. Aprikian.** 1997. Characterization of intracellular calcium mobilization by bombesin-related neuropeptides in PC-3 human prostate cancer cells. Prostate **31**:53–60.
39. **Hanke, J. H., J. P. Gardner, R. L. Dow, P. S. Changelian, W. H. Brissette, E. J. Weringer, B. A. Pollok, and P. A. Connelly.** 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. **271**:695–701.
40. **Heisler, L. E., A. Evangelou, A. M. Lew, J. Trachtenberg, H. P. Elsholtz, and T. J. Brown.** 1997. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol. Cell. Endocrinol. **126**:59–73.
41. **Hellmich, M. R., J. F. Battey, and J. K. Northup.** 1997. Selective reconstitution of gastrin-releasing peptide receptor with G alpha q. Proc. Natl. Acad. Sci. USA **94**:751–756.
42. **Hellmich, M. R., K. L. Ives, V. Udupi, M. S. Soloff, G. H. J. Greeley, B. N. Christensen, and C. M. J. Townsend.** 1999. Multiple protein kinase pathways are involved in gastrin-releasing peptide receptor-regulated secretion. J. Biol. Chem. **274**:23901–23909.
43. **Hoosein, N. M.** 1998. Neuroendocrine and immune mediators in prostate cancer progression. Front. Biosci. **3**:D1274–D1279.
44. **Hoosein, N. M., C. J. Logothetis, and L. W. Chung.** 1993. Differential effects of peptide hormones bombesin, vasoactive intestinal polypeptide and somatostatin analog RC-160 on the invasive capacity of human prostatic carcinoma cells. J. Urol. **149**:1209–1213.
45. **Ikonen, T., J. J. Palvimo, P. J. Kallio, P. Reinikainen, and O. A. Janne.** 1994. Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation. Endocrinology **135**:1359–1366.
46. **Jiang, Y., W. Ma, Y. Wan, T. Kozasa, S. Hattori, and X. Y. Huang.** 1998. The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. Nature **395**:808–813.
47. **Jui, H. Y., R. J. Tseng, X. Wen, H. I. Fang, L. M. Huang, K. Y. Chen, H. J. Kung, D. K. Ann, and H. M. Shih.** 2000. Protein-tyrosine phosphatase D1, a potential regulator and effector for Tec family kinases. J. Biol. Chem. **275**:41124–41132.
48. **Lee, L. F., J. S. Haskill, N. Mukaida, K. Matsushima, and J. P. Ting.** 1997. Identification of tumor-specific paclitaxel (Taxol)-responsive regulatory elements in the interleukin-8 promoter. Mol. Cell. Biol. **17**:5097–5105.
49. **Li, Z., M. I. Wahl, A. Eguinoa, L. R. Stephens, P. T. Hawkins, and O. N. Witte.** 1997. Phosphatidylinositol 3-kinase-gamma activates Bruton's tyrosine kinase in concert with Src family kinases. Proc. Natl. Acad. Sci. USA **94**:13820–13825.
50. **Lin, W., H. W. Kao, D. Robinson, H. J. Kung, C. W. Wu, and H. C. Chen.** 2000. Tyrosine kinases and gastric cancer. Oncogene **19**:5680–5689.
51. **Logothetis, C., and N. Hoosein.** 1992. The inhibition of the paracrine progression of prostate cancer as an approach to early therapy of prostatic carcinoma. J. Cell. Biochem. Suppl. **16H**:128–134.
52. **Ma, Y. C., J. Huang, S. Ali, W. Lowry, and X. Y. Huang.** 2000. Src tyrosine kinase is a novel direct effector of G proteins. Cell **102**:635–646.
53. **Ma, Y. C., and X. Y. Huang.** 1998. Identification of the binding site for Gqalpha on its effector Bruton's tyrosine kinase. Proc. Natl. Acad. Sci. USA **95**:12197–12201.
54. **Mao, J., W. Xie, H. Yuan, M. I. Simon, H. Mano, and D. Wu.** 1998. Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13. EMBO J. **17**:5638–5646.
55. **Markwalder, R., and J. C. Reubi.** 1999. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res. **59**:1152–1159.
56. **Milovanovic, S. R., S. Radulovic, K. Groot, and A. V. Schally.** 1992. Inhibition of growth of PC-82 human prostate cancer line xenografts in nude mice

by bombesin antagonist RC-3095 or combination of agonist [D-Trp6]-luteinizing hormone-releasing hormone and somatostatin analog RC-160. Prostate **20**:269–280.

57. **Miron, L.** 1996. The hormonal and chemotherapy of prostatic cancer. Rev. AQ: R Med. Chir. Soc. Med. Nat. IASI **100**:37–43.
58. **Nagabhushan, M., C. M. Miller, T. P. Pretlow, J. M. Giaconia, N. L. Edgehouse, S. Schwartz, H. J. Kung, W. de Vere, P. H. Gumerlock, M. I. Resnick, S. B. Amini, and T. G. Pretlow.** 1996. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res. **56**:3042–3046.
59. **Nanberg, E., and E. Rozengurt.** 1988. Temporal relationship between inositol polyphosphate formation and increases in cytosolic Ca2+ in quiescent 3T3 cells stimulated by platelet-derived growth factor, bombesin and vasopressin. EMBO J. **7**:2741–2747.
60. **Nazareth, L. V., and N. L. Weigel.** 1996. Activation of the human androgen receptor through a protein kinase A signaling pathway. J. Biol. Chem. **271**: 19900–19907.
61. **Nelson, J., M. Donnelly, B. Walker, J. Gray, C. Shaw, and R. F. Murphy.** 1991. Bombesin stimulates proliferation of human breast cancer cells in culture. Br. J. Cancer **63**:933–936.
62. **Offermanns, S., E. Heiler, K. Spicher, and G. Schultz.** 1994. Gq and G11 are concurrently activated by bombesin and vasopressin in Swiss 3T3 cells. FEBS Lett. **349**:201–204.
63. **Parsons, J. T., K. H. Martin, J. K. Slack, J. M. Taylor, and S. A. Weed.** 2000. Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene **19**:5606–5613. ,
64. **Patel, K. V., and M. P. Schrey.** 1990. Activation of inositol phospholipid signaling and Ca2+ efflux in human breast cancer cells by bombesin. Cancer Res. **50**:235–239.
65. **Qiu, Y., and H. J. Kung.** 2000. Signaling network of the Btk family kinases. Oncogene **19**:5651–5661.
66. **Qiu, Y., D. Robinson, T. G. Pretlow, and H.-J. Kung.** 1998. Etk/Bmx, a tyrosine kinase with a plekstrin-homology domain, is an effector of phosphatidylinositol 3′-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc. Natl. Acad. Sci. USA **95**:3644–3649.
67. **Rawlings, D. J., A. M. Scharenberg, H. Park, M. I. Wahl, S. Lin, R. M. Kato, A. C. Fluckiger, O. N. Witte, and J. P. Kinet.** 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science **271**: 822–825.
68. **Reiske, H. R., S. C. Kao, L. A. Cary, J. L. Guan, J. F. Lai, and H. C. Chen.** 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. J. Biol. Chem. **274**:12361–12366.
69. **Richardson, A., and T. Parsons.** 1996. A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. Nature **380**:538–540. (Erratum, **381**:810.)
70. **Robinson, D., F. He, T. Pretlow, and H. J. Kung.** 1996. A tyrosine kinase profile of prostate carcinoma. Proc. Natl. Acad. Sci. USA **93**:5958–5962.
71. **Rodriguez-Fernandez, J. L.** 1999. Why do so many stimuli induce tyrosine phosphorylation of FAK? Bioessays **21**:1069–1075.
72. **Rodriguez-Fernandez, J. L., and E. Rozengurt.** 1998. Bombesin, vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine induce focal adhesion kinase activation in intact Swiss 3T3 cells. J. Biol. Chem. **273**:19321–19328.
73. **Rozengurt, E.** 1983. Growth factors, cell proliferation and cancer: an overview. Mol. Biol. Med. **1**:169–181.
74. **Rozengurt, E.** 1991. Neuropeptides as cellular growth factors: role of multiple signalling pathways. Eur. J. Clin. Invest. **21**:123–134.
75. **Rozengurt, E.** 1998. Signal transduction pathways in the mitogenic response to G protein-coupled neuropeptide receptor agonists. J. Cell. Physiol. **177**: 507–517.
76. **Sadar, M. D.** 1999. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J. Biol. Chem. **274**:7777–7783.
77. **Salazar, E. P., and E. Rozengurt.** 1999. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. J. Biol. Chem. **274**:28371–28378.
78. **Sato, N., M. E. Gleave, N. Bruchovsky, P. S. Rennie, S. L. Goldenberg, P. H. Lange, and L. D. Sullivan.** 1996. Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model. J. Steroid Biochem. Mol. Biol. **58**:139–146.
79. **Schally, A. V., A. M. Comaru-Schally, A. Plonowski, A. Nagy, G. Halmos, and Z. Rekasi.** 2000. Peptide analogs in the therapy of prostate cancer. Prostate **45**:158–166.
80. **Seethalakshmi, L., S. P. Mitra, P. R. Dobner, M. Menon, and R. E. Carraway.** 1997. Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. Prostate **31**:183–192.
81. **Sehgal, I., S. Powers, B. Huntley, G. Powis, M. Pittelkow, and N. J. Maihle.** 1994. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc. Natl. Acad. Sci. USA **91**:4673–4677.
82. **Spiotto, M. T., and T. D. Chung.** 2000. STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. Prostate **42**:186–195.
83. **Sramkoski, R. M., T. G. Pretlow, J. M. Giaconia, T. P. Pretlow, S. Schwartz, M. S. Sy, S. R. Marengo, J. S. Rhim, D. Zhang, and J. W. Jacobberger.** 1999. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell. Dev. Biol. Anim. **35**:403–409.
84. **Tamagnone, L., I. Lahtinen, T. Mustonen, K. Virtaneva, F. Francis, F. Muscatelli, R. Alitalo, C. I. Smith, C. Larsson, and K. Alitalo.** 1994. BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. Oncogene **9**:3683–3688.
85. **Tsai, Y. T., Y. H. Su, S. S. Fang, T. N. Huang, Y. Qiu, Y. S. Jou, H. M. Shih, H. J. Kung, and R. H. Chen.** 2000. Etk, a Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. Mol. Cell. Biol. **20**:2043–2054.
86. **Tsukada, S., D. C. Saffran, D. J. Rawlings, O. Parolini, R. C. Allen, I. Klisak, R. S. Sparkes, H. Kubagawa, T. Mohandas, and S. Quan.** 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell **72**:279–290.
87. **van der Kwast, T. H., J. Schalken, D. W. J. Ruizeveld, C. C. van Vroonhoven, E. Mulder, W. Boersma, and J. Trapman.** 1991. Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J. Cancer **48**:189–193.
88. **Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, and M. Bobrow.** 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature **361**:226–233. (Erratum, **364**:362.)
89. **Viallet, J., and D. C. Ihde.** 1989. Systemic therapy for small-cell lung cancer: old themes replayed, new ones awaited. J. Clin. Oncol. **7**:985–987.
90. **Viallet, J., Y. Sharoni, H. Frucht, R. T. Jensen, J. D. Minna, and E. A. Sausville.** 1990. Cholera toxin inhibits signal transduction by several mitogens and the in vitro growth of human small-cell lung cancer. J. Clin. Invest. **86**:1904–1912.
91. **Voeller, H. J., G. Wilding, and E. P. Gelmann.** 1991. v-rasH expression confers hormone-independent in vitro growth to LNCaP prostate carcinoma cells. Mol. Endocrinol. **5**:209–216.
92. **Wang, B., J. X. Zou, B. Ek-Rylander, and E. Ruoslahti.** 2000. R-Ras contains a proline-rich site that binds to SH3 domains and is required for integrin activation by R-Ras. J. Biol. Chem. **275**:5222–5227.
93. **Wen, Y., M. C. Hu, K. Makino, B. Spohn, G. Bartholomeusz, D. H. Yan, and M. C. Hung.** 2000. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res. **60**: 6841–6845.
94. **Woll, P. J.** 1991. Neuropeptide growth factors and cancer. Br. J. Cancer **63**:469–475.
95. **Xue, L. Y., Y. Qiu, J. He, H. J. Kung, and N. L. Oleinick.** 1999. Etk/Bmx, a PH-domain containing tyrosine kinase, protects prostate cancer cells from apoptosis induced by photodynamic therapy or thapsigargin. Oncogene **18**: 3391–3398.
96. **Yan, G., Y. Fukabori, S. Nikolaropoulos, F. Wang, and W. L. McKeehan.** 1992. Heparin-binding keratinocyte growth factor is a candidate stromal-to-epithelial-cell andromedin. Mol. Endocrinol. **6**:2123–2128.
97. **Yeh, S., H. K. Lin, H. Y. Kang, T. H. Thin, M. F. Lin, and C. Chang.** 1999. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc. Natl. Acad. Sci. USA **96**:5458–5463.
98. **Zachary, I., and E. Rozengurt.** 1987. Identification of a receptor for peptides of the bombesin family in Swiss 3T3 cells by affinity cross-linking. J. Biol. Chem. **262**:3947–3950.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB263458
ADB282174
ADB270704
ADB282196
ADB264903
ADB268484
ADB282253
ADB282115
ADB263413
ADB269109
ADB282106
ADB262514
ADB282264
ADB256789
ADB251569
ADB258878
ADB282275
ADB270822
ADB282207
ADB257105
ADB281673
ADB254429
ADB282110
ADB262549
ADB268358
ADB257359
ADB265810
ADB282111
ADB273020
ADB282185
ADB266340
ADB262490
ADB266385
ADB282181
ADB262451
ADB266306
ADB260298
ADB269253
ADB282119
ADB261755
ADB257398
ADB267683
ADB282231
ADB234475
ADB247704
ADB258112
ADB267627
ADB282838
ADB233092
ADB263929
ADB282182
ADB257136
ADB282227
ADB282177
ADB263548
ADB246535
ADB282826
ADB282127
ADB271165
ADB282112
ADB255775
ADB265599
ADB282098
ADB232738
ADB243196
ADB257445
ADB267547
ADB277556
ADB239320
ADB253648
ADB282171
ADB233883
ADB257696
ADB232089
ADB240398
ADB261087
ADB249593
ADB264542
ADB282216
ADB261617
ADB269116

Exel