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Ras Signaling in Prostate Cancer Progression

Michael J. Weber* and Daniel Gioeli

Department of Microbiology and Cancer Center, University of Virginia Health System, Charlottesville, Virginia 22908

Abstract When prostate cancer is first detected it generally is dependent on the presence of androgens for growth, and responds to androgen ablation therapies. However, the disease often recurs in a disseminated and apparently androgen independent (AI) form, and in this state is almost invariably fatal. Considerable evidence indicates that the Androgen receptor (AR) continues to be required even in androgen independent (AI) disease. Thus, a key to understanding hormone independent prostate cancer is to determine the mechanism(s) by which the AR can function even in the absence of physiologic levels of androgen. In this article, we argue that growth factors and receptors that utilize Ras family members drive prostate cancer progression to a state of androgen hypersensitivity; and that post-translational modifications (e.g., phosphorylations) of transcriptional cofactors might be responsible for modulating the function of the AR so that it is active even at low concentrations of androgen. J. Cell. Biochem. 91: 13–25, 2004.

Key words: androgen receptor; signal transduction; MAP kinase

PROGRESSION TO HORMONE "INDEPENDENCE"

The normal development, growth, and survival of the prostate epithelium are regulated both by androgen and by the paracrine production of growth factors by the stroma. Similar regulatory interactions between androgens and growth factors also occur in prostate cancer. Stimulation of prostate cancer cells with growth factors can diminish the requirement for androgen, and the expression of these growth factors and receptors increases as prostate cancer progresses toward decreasing dependence on androgen [Scher et al., 1995; Culig et al., 2000, 2002b]. However, even in advanced prostate cancer, that is refractory to hormone ablation therapy, the androgen receptor (AR) continues to be expressed, and is required for cell growth.

Prostate cancer initially requires androgen for growth, and responds to hormone ablation strategies (castration and/or anti-androgens). These are first-line treatments for locally advanced and metastatic disease. However, the disease almost invariably progresses to a state of reduced hormone dependence. Whereas surgery is curative for locally confined prostate cancer, there are no effective treatments for metastatic prostate cancer, once androgen dependence is lost.

When prostate cancer progresses it is variously called "recurrent," or "hormone refractory" (because it is resistant to hormone ablation therapy), or "androgen independent." We believe that advanced prostate cancers often are not fully independent of androgen, but have become sensitive to very low levels of androgen. These cancers may appear to be "androgen independent" clinically, because hormone ablation therapies do not eliminate all traces of androgen. However, at the molecular level they still may depend on androgen and on the AR. This is a source of semantic confusion, because the term "androgen independent" is used in the literature, regardless of whether the cells are completely or only partially androgen independent. In this communication, we will use the abbreviation "AI" for "Androgen Independent," with the understanding that the cells may actually be responsive to very low levels of

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*Correspondence to: Michael J. Weber, Department of Microbiology, University of Virginia Health System, PO Box 800734, Charlottesville, VA 22908.
E-mail: mjw@virginia.edu

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androgen rather than being completely independent of the steroid. Nevertheless, this progression is of profound medical significance since it results in the loss of the only effective treatment for disseminated disease, and thus the recurrent cancer is almost invariably fatal.

Androgen "Independent" Prostate Cancer Still Requires the AR

Even though advanced prostate cancer displays minimal androgen dependence, it long has been suspected that the AR continues to be required in recurrent prostate cancer even when androgen no longer appears necessary. The AR often is overexpressed or mutated in advanced disease, implying a selective pressure to maintain AR function [Gelmann, 2002]. Recent work from the Tindall [Zegarra-Moro et al., 2002] and Klocker [Eder et al., 2002] laboratories confirms the continuing requirement for the AR: ablation of the AR by hammerhead ribozyme expression, or antibody injection, or antisense, inhibits cell growth in LNCaP cells and in their derivatives that are androgen "independent." Thus, advanced prostate cancer may appear to be "independent" of androgen, but it is not independent of the AR. The AR dependent regulatory mechanisms are subverted, not bypassed.

The AR compensates for a deficiency of androgen in several ways in recurrent prostate cancer. In some cases, the AR accumulates mutations that broaden its specificity for ligand, so that it now becomes activated by other ligands such as estrogen—and even by anti-androgens [Culig et al., 2002b; Gelmann, 2002; Isaacs et al., 2002]. Overexpression of transcriptional coactivators or of the AR itself also accompanies prostate cancer progression in some cases, and this too facilitates the activity of the AR [Yeh et al., 1999; Gregory et al., 2001b; Comuzzi et al., 2003]. Finally, the AR can be activated functionally in response to signal transduction from growth factors, as described above [Culig et al., 1994; Ikonen et al., 1994; Nazareth and Weigel, 1996; Reinikainen et al., 1996; Weigel, 1996; Sadar, 1999; Culig et al., 2000, 2002a,b; Rowan et al., 2000a; Steiner et al., 2003]. These compensatory mechanisms are not mutually exclusive, and are likely to be mutually reinforcing. This article focuses on the mechanisms by which growth factor signaling alters AR function because this is a mechanism that is likely to play a role in at least half of advanced prostate cancers, it is subject to therapeutic intervention, and it provides an opportunity to understand how Ras signaling can integrate with the functions of nuclear receptors.

Ras-Mediated Signal Transduction Drives Decreased Androgen Dependence

Increases in autocrine and paracrine growth factor loops are among the most commonly reported changes correlated with progression of prostate cancer from localized and androgen dependent to disseminated and AI. EGF, TGF-α, KGF, bFGF, and IGF-I as well as their cognate ligands have all been reported to be overexpressed in advanced prostate cancer [Culig et al., 1994, 2000, 2002b]. Could the signaling pathways activated by these growth factor receptors be causal in driving progression to androgen "independence?" Numerous reports indicate that the answer is yes. Growth factor stimulation has been reported to render ARE-driven promoters hypersensitive to, or "independent" of, androgen [Culig et al., 1994, 2000, 2002a,b; Ikonen et al., 1994; Nazareth and Weigel, 1996; Weigel, 1996; Weigel and Zhang, 1998; Steiner et al., 2003]. Moreover, overexpression of HER2 renders growth of androgen-dependent prostate cancer cells less dependent on androgen [Craft et al., 1999b]. The diversity of these changes in autocrine and paracrine signaling predicts that, at least in the context of prostate cancer, attempts to utilize a single receptor/ligand pair as a therapeutic target will not be generally effective [Mellinghoff et al., 2002]. To identify optimal targets for therapy, we believe it will be necessary to identify the downstream signaling intermediates that are shared by these diverse receptors and ligands.

Ras Activation Correlates With Prostate Cancer Progression

Ras activation is a component of the signaling pathways for virtually all the receptors shown to be upregulated in advanced PCa. Although Ras is infrequently mutated in prostate cancer, we hypothesize that wild-type Ras is chronically activated by autocrine and paracrine growth factor stimulation in prostate cancer. It thus represents a "node" or "intersection point" for these diverse ligands and receptors and therefore may be an appropriate target for therapeutic intervention.

To test whether Ras might be activated during prostate cancer progression in patients, we examined 82 paraffin thin sections from primary and metastatic prostate tumor specimens with an activation-state specific phospho-MAP kinase antibody [Gioeli et al., 1999, 2001]. Activation of MAP kinase in this case was used as a surrogate for Ras activation, because it is not possible to directly measure Ras activity in these samples. We found that the frequency and extent of MAP kinase activation increases with increasing tumor stage and grade. Moreover, we have two anecdotal cases where patients presented with androgen-dependent cancer that was negative for active MAP kinase; but when the disease recurred after androgen ablation, the recurrent tumors were positive for active MAP kinase. Finally, we find that in the hormone dependent CWR22 prostate cancer xenograft, although the tumor regresses after castration, its recurrence correlates with upregulation of phospho-MAP kinase (unpublished). It thus is clear that activation of the MAP kinase pathway correlates with prostate cancer progression in a variety of settings.

Ras Activation Is Sufficient and may be Necessary for Reduced Androgen Dependence

More recently we have demonstrated that Ras activation can play a *causal* role in moving prostate cancer cells toward decreased hormone dependence and increased malignant phenotype [Bakin et al., 2003b]. Building on the seminal work of Gelmann et al. [Voeller et al., 1991], we expressed activated V12 H-Ras effector loop mutants in LNCaP cells, which are largely dependent on androgen for growth. The Ras effector loop mutants preferentially activate one set of effectors vs. another, as described below. We then evaluated the effects of these mutants on androgen dependence of growth and tumorigenicity. We found that some of these activated Ras mutants dramatically reduced the androgen requirement of these cells with respect to growth and also for expression of the PSA protein and mRNA. The mutants that caused these biological changes were the ones that caused an intrinsic activation of the MAP kinase pathway under basal, serum-free conditions (T35S and E37G). This correlates the MAP kinase pathway with changes in androgen dependence in cell culture. Expression of Ras also increased the ability of LNCaP cells to form tumors and to resist regression after castration.

Collectively, these findings show that activation of Ras signaling is sufficient for progression of LNCaP cells toward androgen independence, with respect to growth, gene expression, and tumorigenicity. Moreover, this correlates with activation of MAP kinase signaling.

Conversely, we have found that activation of Ras signaling may be necessary for progression in at least one model: expression of a dominant negative N17 Ha-Ras actually can restore androgen dependence to an androgen "independent" cell line. We utilized C4-2 cells, which were derived by Leland Chung and colleagues from LNCaP cells by serial passage in castrated mice [Thalmann et al., 2000]. Note that the selective pressure during the derivation of these cells was not explicitly for any aspect of Ras signaling. C4-2 cells demonstrate decreased androgen dependence of growth both in vitro and in vivo, increased tumorigenicity in vivo and the ability to grow in soft agarose (anchorage independence) compared to the parental LNCaP cells. We expressed the dominant negative Ras under the control of a tetracycline-inducible promoter in C4-2 prostate cancer cells. When implanted in nude mice, the C4-2 derivatives continued to grow after castration, or when N17 Ras was induced with Doxycycline. However, when the mice were castrated and were also treated with Doxycycline to induce N17Ras, the tumors regressed, in most cases completely.

In summary, our findings and those previously published clearly implicate Ras signaling in progression to androgen independence:

- *Ras signaling correlates with progression:* Overexpression of growth factors and receptors that utilize Ras, and activation of MAP kinase, correlate with prostate cancer progression, in patients. Recurrent CWR22 tumors displayed activated MAP kinase.
- *Ras signaling is sufficient for progression:* Expression of activated Ras makes LNCaP cells less dependent on androgen.
- *Ras signaling is necessary for progression:* Expression of dominant negative Ras restores androgen dependence to C4-2 cells.

Effectors and Partners of Ras

The Ras subfamily of small GTP-binding proteins control signal transduction between the membrane and the nucleus [Bar-Sagi, 2001;

Berthiaume, 2002; Ehrhardt et al., 2002]. They are activated when bound to GTP and inactive when bound to GDP. These states are regulated by the balance between the intrinsic GTPase activity of the proteins, their interactions with inactivating proteins that accelerate their GTPase activity (GAPs—GTPase activating proteins) and with activating proteins that regulate the exchange of GDP for GTP (GEFs—GTP exchange factors). They thus can function both as molecular switches, and as timers. The founding members of the Ras subfamily, H-Ras and K-Ras, were discovered as oncogenes and most of the related proteins also have oncogenic activity when overexpressed in activated form in the appropriate cell background. Most of our knowledge about the biochemistry of Ras signaling is based on analysis of H-Ras; however, K-Ras is the isoform that is most frequently found mutated in human cancers [Bos, 1989; Lowy and Willumsen, 1993].

The paradigmatic signaling activity of Ras involves the activation of a GEF by a receptor tyrosine kinase, the subsequent activation of Ras, the recruitment of Raf to the plasma membrane and its subsequent activation, and the activation of MEK and then MAP kinase (Fig. 1). However, over the last several years it has become increasingly evident that Ras proteins function as part of a network of signaling molecules that include kinases, adapters, other Ras proteins, as well as the GEFs and GAPs.

H-Ras is a multi-effector signaling molecule that has been shown to engage at least a half-dozen signaling pathways. The best studied with respect to malignant transformation are Raf/MEK/MAP kinase, PI3 kinase, and Ral-GDS (Fig. 1). Recently, the interaction of Ras with NORE1, a member of the RASSF1 tumor suppressor family, has been shown to regulate apoptosis [Feig and Buchsbaum, 2002]. Thus, Ras effectors include regulators of both growth and survival. It seems very likely that the ability of Ras to trigger either growth or apoptosis depends on the balance of interactions between pro-growth, pro-survival, and pro-death effectors [Carson et al., 1999; Feig and Buchsbaum, 2002].

The signaling activity of Ras GTPases occurs not only through engagement of direct effectors, but also by the recruitment of other GTPases, especially other members of the Ras sub-family (e.g., Rap), and members of the Rho sub-family (e.g., RhoA, Rac1, cdc42). This "hierarchical

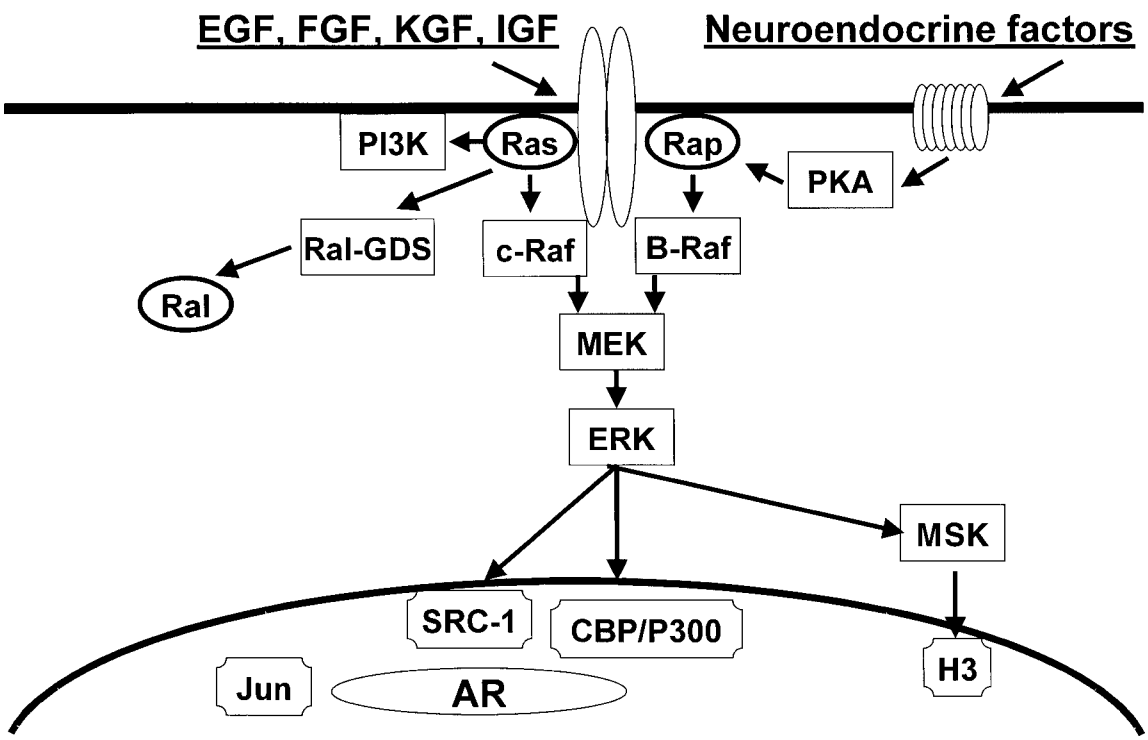

Fig. 1. Growth factor signaling through Ras proteins regulates the androgen receptor (AR).

networking" between different Ras isoforms is controlled in part by interactions with GEFs, GAPs, and effectors [Walsh and Bar-Sagi, 2001; Ward et al., 2001; Ehrhardt et al., 2002; Innocenti et al., 2002; Weijzen et al., 2002; Weston and Davis, 2002]. For example, H-Ras, K-Ras, and N-Ras all share with the Raps the ability to bind to both c-Raf-1 and B-Raf. This may explain why Rap1 was originally isolated as an *inhibitor* of transformation by K-Ras. However, Rap1 can be activated by different GEFs that in turn are responsive to different agonists (e.g., cAMP and Ca) and may be negatively regulated by different GAPs. In this way, Rap1 could cooperate with Ras in regulating the context and the timing of signaling through Raf. Indeed, Rap is reported to control the late phase of MAP kinase activation in NGF-treated neurons [Stork and Schmitt, 2002] and we showed that in prostate cancer cells Rap1 is responsible for the synergism between EGF and agonists that elevate cAMP (e.g., PTHrP and epinephrine) [Chen et al., 1999]. Similarly, many of the GEFs bind more than one Ras member, and thus can serve as regulators of the balance between activation of one or another.

The dominant players, and the specifics of the way they are networked is very much dependent on cellular context. Indeed, a recent paper postulates that whereas PI3K and Raf are the predominant Ras effectors in rodent cells, in human cells the predominant player in oncogenesis is Ral [Hamad et al., 2002].

A schematic of some of the networking of Ras signals is shown in Figure 2. Why so much complexity? In at least some cases, it appears that each signaling molecule has a regulatory "territory," for which it is responsible, as well as a preferred "portal" for stimulation, and that this division of labor is necessary to coordinate very complex responses to extracellular signals (Fig. 3). For example, activation of PI3 kinase is associated with anti-apoptotic signaling (through Akt) and control of protein synthesis (through mTor). Activation of the Rho family is necessary for growth factors to stimulate changes in cell shape and movement. Although these various Ras partners and effectors communicate with each other continuously, through various feedback loops, regulating the expression and activity of various Ras family isoforms has allowed the identification of specific subsets of signals that control different aspects of tumorigenesis in different cellular contexts. For example, we have previously reported that in LNCaP cells dominant negative N17-Ras blocks the ability of EGF to turn on the MAP kinase pathway; whereas dominant negative N17-Rap1 only blocks the ability of EGF and cAMP to synergize with each other [Chen et al., 1999; Bakin et al., 2003b].

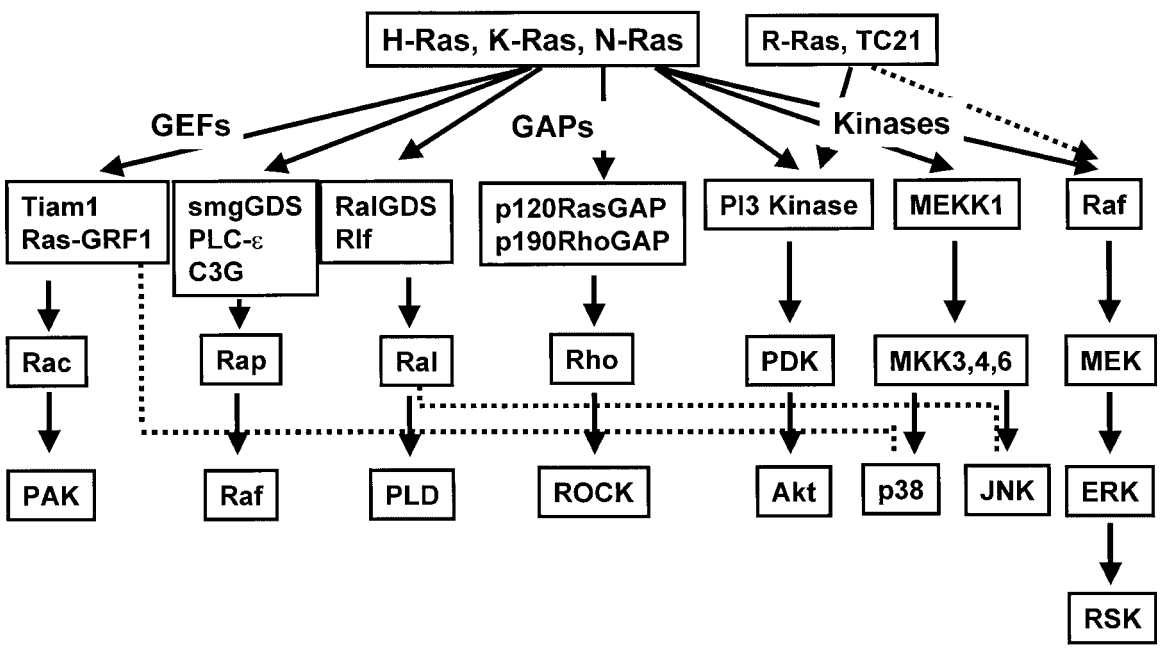

Fig. 2. Hierarchical signaling networks of Ras family members, regulators, and effectors.

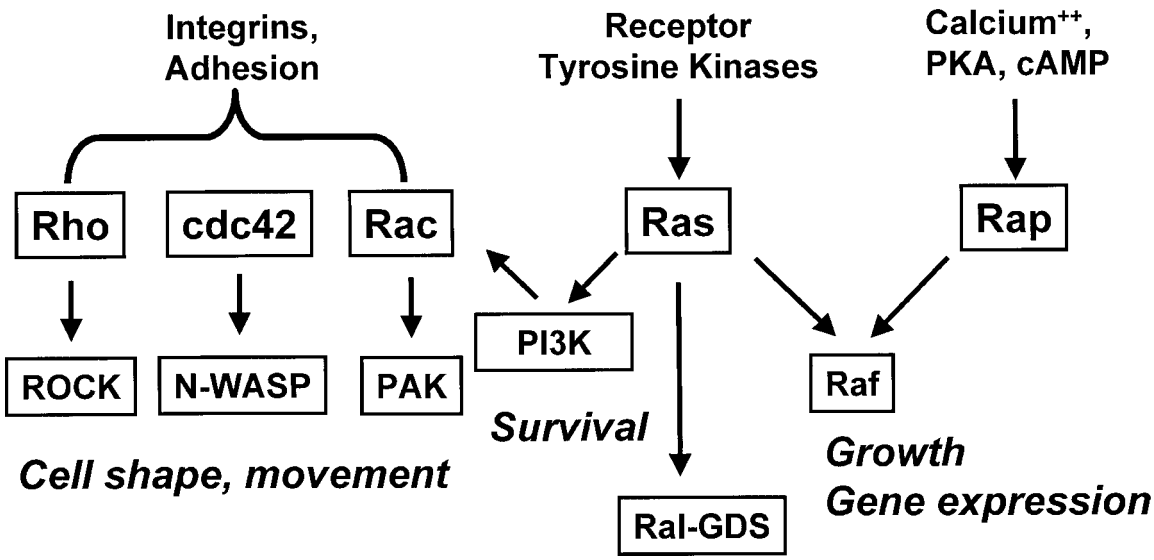

Fig. 3. Ras family signaling: multiple inputs and outputs.

Role of MAP Kinase Signaling

For the following reasons, we suspect that the Raf/MEK/ERK pathway plays a critical role in the modulation of AR activity in response to Ras:

- MAP kinase activation correlated with prostate cancer progression in patient samples [Gioeli et al., 2001].
- The Ras effector loop mutants that had the greatest biological effect on LNCaP cells in vitro were the mutants that activated MAP Kinase [Bakin et al., 2003a,b].
- All androgen "independent" xenografts displayed elevated phospho-MAP kinase, regardless of whether their androgen independence was selected by serial passage, or generated by expressing Ras (unpublished).
- Co-transfection of a mutationally activated MEK will drive the AR-dependent expression of a reporter plasmid controlled by the PSA promoter or by tandem AREs [androgen response elements] (unpublished).
- Inhibition of MEK with PD98059 blunts the ability of androgen to stimulate expression of an ARE-driven reporter (unpublished).

Although MAP kinase signaling is an important component of Ras-driven progression to androgen independence, it is clear that it is not the whole story:

- Whereas N17 Ras expression combined with castration induced tumor regression our preliminary data suggest that MEK inhibition combined with androgen ablation is only cytostatic.
- Conversely, although we can readily isolate LNCaP clones expressing Ras effector loop mutants that activate MAP kinase, we have been unable to stably express mutationally activated MEK in LNCaP (unpublished).

These data indicate that some aspect of Ras signaling is necessary for growth, survival, and androgen responsiveness of prostate cancer cells, beyond just the MAP kinase pathway.

Modulating AR Function in Androgen "Independent" Prostate Cancer

As with other steroid receptors, the AR consists of an N-terminal transcriptional regulatory domain (AF-1) that can function in the absence of ligand, a DNA binding domain, a hinge region, and a C-terminal ligand-binding domain that also is associated with a second transcriptional regulatory function (AF-2). In its unliganded state, the AR is sequestered with chaperones and is not concentrated in the nucleus. Upon ligand binding, a nuclear import signal is exposed and the receptor becomes concentrated in the nucleus where it binds DNA, homodimerizes in a reaction that involves interactions between the N- and C-termini, and interacts with a constellation of transcriptional

coregulators, transcription factors, and components of the basal transcription machinery [Langley et al., 1995; Bubulya et al., 1996, 2000, 2001; Doesburg et al., 1997; Aarnisalo et al., 1998; Fronsdal et al., 1998; Tillman et al., 1998; Wise et al., 1998; Nessler-Menardi et al., 2000; Reutens et al., 2001; Wang et al., 2001; Gelmann, 2002; Heinlein and Chang, 2002; Kotaja et al., 2002a,b; McKenna and O'Malley, 2002a,b; Comuzzi et al., 2003]. Ras-mediated signaling could modulate AR function at any of these steps, and it will be important to determine where that regulatory intersection occurs.

Role of AR Phosphorylation

The sensitivity of the Estrogen receptor (ER) to estradiol is regulated in part by the phosphorylation of the ER [Lannigan, 2003]. The ER is directly phosphorylated by ERK and RSK2 (which is downstream of MAP kinase) [Clark et al., 2001]. These phosphorylations render the ER hypersensitive to estradiol. By analogy, we predicted that the AR would be a direct substrate of MAP kinase signaling, and wished to determine whether AR phosphorylation might regulate androgen sensitivity.

This hypothesis is supported by a substantial body of literature that suggests the AR is regulated—directly or indirectly—by signal transduction cascades involving protein kinases and phosphorylation [Culig et al., 1994, 2000, 2002a,b; Ikonen et al., 1994; Nazareth and Weigel, 1996; Weigel, 1996; Weigel and Zhang, 1998; Sadar, 1999; Steiner et al., 2003]. Previous studies have inferred candidate phosphorylation sites on the AR, by in vitro phosphorylation reactions and/or by identifying kinase consensus sites and then mutagenizing them. Sites so identified include serines 81, 94, 213, 515, 650, and 791 [Jenster et al., 1994; Zhou et al., 1995; Yeh et al., 1999; Wen et al., 2000; Lin et al., 2001]. (All AR amino acid numbers in this paper are based on NCB Accession no. AAA51729 [Lubahn et al., 1988a,b]). However, these determinations, although a useful first step, are not definitive because in vitro kinase reactions are often not selective, and mutagenesis can alter the phosphorylations on sites distinct from the ones mutagenized. Recently, Ser 308 was directly identified as a phosphorylation site in Baculovirus over-expressed AR using mass spectrometry [Zhu et al., 2001]. This is the first site identified in living cells either by mass spectrometry or by in vivo metabolic labeling. Because unequivocally identifying the in vivo sites of AR phosphorylation is fundamental to understanding the interactions of the AR and cell signaling, we undertook an extensive study of AR phosphorylation to explore regulated changes in AR phosphorylation as a possible mechanism for activation/sensitization of AR-dependent gene expression by cell surface receptors and their downstream signaling effectors.

In what turned out to be a formidable technical challenge, we mapped the seven major phosphorylation sites on the AR in living cells. One site is constitutively phosphorylated, six sites are regulated in response to androgen, and one of these, S650, becomes phosphorylated in response to a number of non-steroid agonists, including EGF, PMA, forskolin [Gioeli et al., 2002], and anisomycin [Gioeli et al., in press]. In collaboration with Bryce Paschal, we found that phosphorylation on this site regulates nuclear export. We also have found that when one androgen induced phosphoryaltion site, S308, is mutated to Alanine, the AR can give a heightened transcriptional response to steroid, as measured by a reporter assay [Gioeli et al., 2002].

Although ERK is capable of phosphorylating AR in vitro on S515 [Yeh et al., 1999], we did not detect phosphorylation on this residue in living cells [Gioeli et al., 2002]. Moreover, the peak of MAP kinase activation following growth factor stimulation occurs around 10–15 min, whereas phosphorylations on the AR occur more slowly, peaking after one or more hours following agonist stimulation [Gioeli et al., 2002]. Addition of a MEK inhibitor did not substantially alter the pattern of AR phosphorylations. However, this does not necessarily mean that the AR is not an in vivo ERK substrate. It is possible that the S515 phosphorylation occurs under conditions we did not investigate, or that the stoichiometry of phosphorylation is low. (A low stoichiometry phosphorylation can be highly significant—it might, e.g., be transitory, yet regulate a key aspect of receptor function.) Thus, it is not resolved whether the AR is a direct substrate for MAP kinase, but the weight of evidence suggests that the AR, in contrast to the ER, is not directly phosphorylated by ERK pathway kinases.

Because we have not found sites that reproducibly alter AR sensitivity to androgen, or found sites that are clearly direct sites of phos-

phorylation by MAP kinase, we hypothesize that Ras-mediated signaling alters AR function by inducing phosphorylation of AR partners, and/or proteins that modify AR in other ways

AR and Its Partners

Transcriptional coregulators control the susceptibility of chromatin to transcription (chromatin remodeling) or the recruitment of the transcriptional machinery (e.g., RNA polymerase-II) or both. The coregulators can be coactivators or corepressors, and the group that has received perhaps the greatest recent attention regulate the acetylation of histones and other components of the transcription machinery, including the AR [Fu et al., 2002; Gaughan et al., 2002]. These are Histone acetyl transferases (HATs) that function as coactivators; and Histone deacetylases (HDACS) that can function as corepressors. However, these enzymes work in concert with ATP-dependent chromatin remodeling (SWI/SNF), arginine methyltransferases (CARM1 and PRMT1), and Histone kinases [Berger, 2002; Geiman and Robertson, 2002].

A simplified generic model for the assembly of a functional transcription unit [Featherstone, 2002] would begin with binding of a transcription factor to a DNA enhancer/promoter recruitment of a histone kinase, and phosphorylation of Histone H3 at Serine 10. A proposed kinase for the S10 phosphorylation is MSK [Soloaga et al., 2003], which is a MAP kinase-activated kinase. H3 phosphorylation in turn triggers events that lead to recruitment of HAT complexes and other chromatin remodeling enzymes, exposure of the TATA box, binding of TBP, exposure of the transcriptional start site, and recruitment of pol-II and other components of the transcription machinery. Whether these events truly occur sequentially, or coordinately, is debatable. But it is clear that an important role in regulating their assembly is played by signal transducing kinases that can phosphorylate histones, coactivators, and the basal transcription machinery. For example, CBP was first described as the partner for the cAMP-regulated transcription factor CREB [Chrivia et al., 1993]. Moreover, it also is a phosphoprotein, and is subject to phosphorylation by PKC, CaM kinase, and others [Goodman and Smolik, 2000].

These proteins are prime suspects in searching for AR regulators that in turn are regulated by signal transduction [Weigel, 1996; Aarnisalo et al., 1998; Fronsdal et al., 1998; Chadee et al., 1999; Font and Brown, 2000; Rowan et al., 2000a,b; Gnanapragasam et al., 2001; Featherstone, 2002; Gaughan et al., 2002; Heinlein and Chang, 2002; McKenna and O'Malley, 2002a,b; Wu et al., 2002; Comuzzi et al., 2003]:

- Several coregulators have been identified as targets of signaling pathways, including the MAP kinase pathway (e.g., SRC-1, CBP, p300, AIB1, GRIP1).
- Knock-out of SRC-1 in mice results in defective growth of the prostate [Xu et al., 1998].
- SRC-1 and TIF2/GRIP1 are overexpressed in recurrent prostate cancers [Gregory et al., 2001b].
- Overexpression of TIF2/GRIP1, ARA55, or ARA70 increase the transcriptional activity of AR in response to low affinity ligands (e.g., DHEA, androstenedione, estradiol) or to low concentrations of DHT [Yeh et al., 1999; Heinlein and Chang, 2002]. We hypothesize that phosphorylation of these coactivators provides an alternative to overexpression as a mechanism for regulating AR.
- p300 mediates IL6 activation of AR, and overexpression of p300 can overcome the ability of MEK-inhibition to block the IL6-simulated transactivation [Debes and Tindall, 2002; Debes et al., 2002; Huang and Tindall, 2002]. AR also interacts physically and functionally with other transcription factors including c-Jun [Bubulya et al., 1996, 2000, 2001; Tillman et al., 1998; Wise et al., 1998].

Chung and colleagues [Yeung et al., 2000] have mapped the PSA promoter to determine the regions that are responsible for the differential basal gene expression between LNCaP and C4-2. They identify both the ARE in the enhancer, and a site with similarity to SP-1 family sites, near the promoter. These data are consistent with the concept that co-activators interacting with AR as well as transcription factors that can directly bind DNA could be involved in progression to decreased androgen dependence.

AR and coactivators are also regulated by other post-translational modifications, such as sumoylation and methylation as well as acetylation [Poukka et al., 2000; Stallcup, 2001; Gaughan et al., 2002]. In this article, we

focused on phosphorylation because our goal is to understand the intersection between Ras signaling—which activates kinase cascades—and the AR. However, it is possible that the targets of phosphorylation could be regulators of these other processes.

We propose that heightened AR activity is generated by Ras-mediated signaling pathways that regulate the AR through modification of transcriptional co-regulators. Consistent with this hypothesis, the data that we have reviewed shows that overexpression or activation of every component of this pathway can decrease the dependence of prostate cancer cells for androgen: Growth factors [Culig et al., 1994, 2002a; Ikonen et al., 1994; de Ruiter et al., 1995; Nazareth and Weigel, 1996; Reinikainen et al., 1996; Weigel, 1996; Darne et al., 1998; Weigel and Zhang, 1998; Dai et al., 2002; Di Lorenzo et al., 2002], Growth factor receptors [Craft and Sawyers, 1998; Abreu-Martin et al., 1999; Craft et al., 1999a,b; Nickerson et al., 2001; Chen and Sawyers, 2002; Mellinghoff et al., 2002], Ras [Voeller et al., 1991; Bakin et al., 2003a,b], co-activators [Gregory et al., 1998, 2001a; Grossmann et al., 2001; Debes et al., 2002a], or AR [Craft and Sawyers, 1998; Abreu-Martin et al., 1999; Craft et al., 1999a,b; Gregory et al., 2001b; Nickerson et al., 2001; Chen and Sawyers, 2002; Mellinghoff et al., 2002]. Thus, if our reasoning is correct, there is not a single mechanism for progression to androgen "independent" prostate cancer, but rather a constellation of mutually reinforcing mechanisms.

Implications for Therapy of Advanced Prostate Cancer

The ideal "target" for anti-cancer therapy has a unique and essential function in the cancer cells. BCR-Abl meets these criteria for CML but may be atypical. It is possible that prostate cancer, where progression is characterized by multiple genetic alterations and by overexpression of multiple growth factors and receptors, is more typical, certainly of solid tumors. It is not known which of these paracrine and autocrine systems is/are of greatest functional significance, or whether they are redundant. Without that information, it becomes impossible to determine which receptor or combination of receptors might make the most appropriate target(s) for therapy, and whether that might differ from one patient to another. HER1 has been a major focus in recent years, but inhibition of this receptor with small molecules has had disappointing therapeutic effects [Dancey and Freidlin, 2003].

The problems associated with functional redundancy of growth factor receptors are inevitably complicated even further by the well established but widely ignored observation that kinase-dead EGF receptor is capable of intracellular signaling, apparently by dimerization with other receptors or kinases [Coker et al., 1994; Wright et al., 1995]. Thus, it is not even certain that an "essential" target (as determined with knockout or dominant-negative methodologies) would be a useful target for a small molecule catalytic inhibitor.

Intracellular signaling may provide more effective targets, because, although redundancy is common, some of the "nodes" where signaling pathways converge, have been identified. The MAP kinase pathway represents one of those sites of regulatory convergence. It is widely believed that the downside of targeting intracellular signaling is that the same regulatory modules are used in multiple functions, and thus that drugs that inhibit these pathways might display widespread mechanism-induced toxicities. It will be important to determine through preclinical studies and clinical trials whether inhibitors of signaling enzymes (e.g., MEK, Raf, and Src) are more substantial than the EGFR inhibitors, and whether they also are more toxic.

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