High Intensity ras Signaling Induces Premature Senescence by Activating p38 Pathway in Primary Human Fibroblasts*

Received for publication, August 6, 2003, and in revised form, October 24, 2003
Published, JBC Papers in Press, October 29, 2003, DOI 10.1074/jbc.M308644200

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Although oncogenic ras plays a pivotal role in neoplastic transformation, it triggers an anti-oncogenic defense mechanism known as premature senescence in normal cells. In this study, we investigated the induction of cellular responses by different expression levels of oncogenic ras in primary human fibroblasts. We found that a moderate, severalfold increase in ras expression promoted cell growth. Further elevation of ras expression initially enhanced proliferation but eventually induced p16INK4A expression and senescence. The induction of these opposing cellular responses by ras signals of different intensity was achieved through differential activation of the MAPK pathways that mediated these responses. Whereas moderate ras activities only stimulated the mitogenic MEK-ERK pathway, high intensity ras signals induced MEK and ERK to higher levels, leading to stimulation of the MKK3/6-p38 pathway, which had been shown previously to act downstream of Ras-MEK to trigger the senescence response. Thus, these studies have revealed a mechanism for the differential effects of ras on cell proliferation. Furthermore, moderate ras activity mediated transformation in cooperation with E6E7 and hTERT, suggesting that a moderate intensity ras signal can provide sufficient oncogenic activities for tumorigenesis. This result also implies that the ability of ras to promote proliferation and oncogenic transformation can be uncoupled with that to induce senescence in cell culture and that the development of tumors with relatively low ras activities may not need to acquire genetic alterations that bypass premature senescence.

Neoplastic transformation is caused by multiple and successive genetic alterations (1), among which the oncogenic activation of ras genes seems to be one of the critical steps. The ras proto-oncogenes encode a family of small GTP-binding proteins that transduce mitogenic signals from the cell surface. Amplification of the ras genes and constitutively active ras mutant alleles has been frequently found in a wide variety of tumors (2), suggesting that the growth signals elicited by ras are crucial for tumorigenesis. Whereas wild-type ras transduces mitogenic signals in response to extracellular stimuli, activated ras provides cells with constitutive growth signals. The key role of activated ras in promoting oncogenic transformation has been established by numerous studies. In both in vitro cell culture systems and animal models, activated ras genes can cooperate with other oncogenic genetic alterations to lead to transformation (3–8). In addition, ras genes have been found to be activated in a variety of carcinogen-induced tumors in animals, suggesting that ras genes are targets of carcinogens and that they are likely to participate in the initiation of neoplastic transformation (9). The transforming activity of activated ras depends on its interaction with multiple downstream effectors, which mediate different aspects of oncogenic transformation (10, 11). One of the best characterized ras downstream effectors is Raf-1 (12–17). Interaction between Ras and Raf-1 leads to the sequential activation of MAP3 kinase kinases MEK1 and MEK2 and MAP kinases (MAPK) ERK1 and ERK2. Activated ERKs have been shown to promote cell proliferation in tumor cells (18–20).

In early passage primary human and rodent cells, however, the outcome of ras activation alone is somewhat different. Expression of activated ras was initially mitogenic but eventually caused a permanent cell cycle arrest in G1 when activation was sustained for a few days (21, 22). The arrest was phenotypically indistinguishable from replicative senescence observed in late passage primary cells, indicating that oncogenic ras induces premature senescence in these otherwise proliferating, early passage cells. The premature senescence induced by ras was accompanied by accumulation of cell cycle inhibitors such as p53, p21WAF1, p16INK4A, and/or p19ARF, decreased expression of cyclin A, and reduced kinase activity of cyclin-dependent protein kinase-2 (21, 22), all of which are characteristics consistent with growth arrest. The ability of ras to induce premature senescence relies on the activation of the Raf-1/MEK/ERK MAPK pathway that also mediates cell proliferation (24, 25). We recently showed that the stress-induced p38 MAPK acted downstream of MEK to mediate ras-induced p16INK4A expression and senescence (26). Upon activation by ras, MEK subsequently stimulates the activity of p38-activating kinases MKK3 and MKK6, resulting in the induction of p38 activity. Constitutive activation of p38 by active MKK3 or

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The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated or extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein kinase kinase; PGK, phosphoglycerate kinase; GFP, green fluorescence protein; ECL, enhanced chemiluminescence; PD, population doubling; PBS, phosphate-buffered saline; BSA, bovine serum albumin; rTα, reverse tetracycline transactivator; SV40, simian virus 40; LT, large T antigen; st, small t antigen; HPV, human papillomavirus; Rh, retinoblastoma; CR1, conserved region 1; CR2, conserved region 2; BrdUrd, bromodeoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
MKK6 is sufficient to induce premature senescence. In addition, \textit{ras} fails to provoke senescence when p38 activity is inhibited, suggesting an essential role of p38 in the induction of senescence response. These findings imply that the p38 pathway, when activated by \textit{ras}, can limit the transforming potential of \textit{ras} in primary cells by inducing premature senescence.

The ability of oncogenic \textit{ras} to induce senescence-like growth arrest as well as proliferation and transformation suggests that mechanisms must exist that regulate the switch in the signal specificity of \textit{ras} in cells. In this study, we attempt to explore these mechanisms. Because the \textit{ras} expression levels vary substantially in human tumors, ranging from several-fold to more than 50-fold over the wild-type levels (6, 27), we investigated the impact of differential expression levels of oncogenic \textit{ras} on cellular responses in primary human fibroblasts. We show that the type of response \textit{ras} triggers in these cells relies on the strength of \textit{ras} expression. Whereas a strong \textit{ras} signal induces premature senescence, moderate \textit{ras} activity promotes cell proliferation and oncogenic transformation. Further studies reveal that \textit{ras} signals of different strength lead to differential activation of separate MAPK pathways that mediate these distinct cellular responses. Moderate \textit{ras} signals stimulate the activity of the mitogenic MEK-ERK pathway but not that of the senescence-inducing MKK3/6-p38 pathway. In contrast, high intensity \textit{ras} signals induce MEK-ERK to a higher degree, which results in activation of the MKK3/6-p38 pathway and increased expression of p16INK4A. These results have therefore provided a molecular mechanism by which oncogenic \textit{ras} can confer opposing effects on proliferation in the same cells.

EXPERIMENTAL PROCEDURES

Cell Culture—BJ human foreskin fibroblasts (a gift from Dr. J. Smith, Baylor College of Medicine) were maintained in minimum essential medium with 10% fetal calf serum, non-essential amino acids, and glutamine. Lin-X A retroviral packaging cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and glutamine.

Retroviral Vectors and Their Transduction—WZL-hygro- and Babe-puro-Ha-RasV12 were obtained from Dr. Lowe (21, 24). \textit{kTERT} cDNA was from Dr. Weinberg (28) and was subcloned into Babe-puro. BJ-st was from Dr. Weinberg (29). LXSNE-E6, -E7, and -E6-E7 were from Dr. Galloway (30). Mutant E7 was generated by site-directed mutagenesis (QuikChange kit, Stratagene). Ha-RasV12 and GAPDH, GFP, E6E7, or SN-E6, -E7, and -E6E7 were from Dr. Xs. Ras mutant E7 was introduced into the E6 and -E7 plasmids by cotransfection with a plasmid carrying the \textit{E7} cDNA fragment and isolated with the formula

\begin{equation}
\text{PD} = \frac{L - W}{W} \times 100,
\end{equation}

where \(L\) and \(W\) indicate the length and width of a tumor, respectively. For each sample, at least 200 cells were counted in random chosen fields for each sample.

**Protein Expression**—3 days after retroviral infection, BJ cells (PD25) transduced with WZL-Hygro-Ha-RasV12, binary vectors for Ha-RasV12, or control vectors were grown in medium containing 0.1% FBS for 24 h before being labeled with 50 \(\mu\)M BrdUrd (Sigma) for 2 h. Cells were harvested and fixed in 70% ethanol at 4°C overnight. Cells were then treated with 2 N HCl, 0.5% Triton X-100 for 30 min at room temperature and neutralized with 0.1 M Na2B4O7 and subsequent fixation with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (BD Biosciences) for 1 h at room temperature. Finally, cells were washed with PBS, 1% BSA, 0.5% Tween 20, resuspended in PBS containing 5 \(\mu\)g/ml propidium iodide, and analyzed by two-dimensional flow cytometry.

**Construction of Cell Lines Expressing Doxycycline-inducible Ha-RasV12**—BJ cells (PD17) were serially infected with Babe-puro-kTERT and a virus expressing rTAT (WZL-Neo-rTAT) (33). WZL-Neo-rTAT was a gift from Dr. R. Maestro (CRO-IIRRCS, National Cancer Institute, Aviano, Italy). Single cell clones were isolated after drug selection, transduced individually with a retrovirus expressing a LacZ reporter under the control of a doxycycline-inducible promoter (Babe-SIN-Hygro-tet-LacZ) and tested for the induction of LacZ expression by doxycycline. The most responsive clone (before transduction of Babe-SIN-Hygro-tet-LacZ) was selected and transduced with a virus expressing doxycycline-inducible Ha-RasV12 (Babe-SIN-Hygro-tet-Ha-RasV12). Transduced cells were purified by selection with 10 \(\mu\)g/ml hygromycin. Single-cell clones were isolated, propagated, and tested for induction of Ha-RasV12 by different concentrations of doxycycline.

**Tumorigenic Assays**—BJ cells (PD10–20) were serially transduced with kTERT and other oncogenes to construct transformed cell lines. For soft agar assays, 10\(^5\) cells at PD30–45 were resuspended in 2 ml of growth medium containing 0.5% low melting temperature agarose (SeaPlaque GTG, FMC BioProducts) and plated onto 2 ml of solidified bottom layer medium containing 0.5% low melting agarose in 6-well plates in triplicates. Cultures were fed every 7–10 days. Colonies were stained with 0.02% Giemsa and counted 4 weeks after plating.

**Proliferation Assays**—5 \(\times\) 10\(^5\) cells at PD30–45 were resuspended in 100 \(\mu\)l of serum-free RPMI 1640 medium and injected subcutaneously into 6–8-week-old female athymic WEHI nude mice (Roden Breeding Colony, The Scripps Research Institute). Tumor size was measured every 4–6 days, and the volume of a tumor was calculated with the formula \(V = (L \times W^2)/2\), where \(L\) and \(W\) indicate the length and width of a tumor, respectively. At least 200 cells were counted in random chosen fields for each sample. Detailed chromosome analysis was performed with 20 GTG-banded metaphase cells of at least the 450–500 band level.

**RESULTS**

**Induction of Premature Senescence Relies on the Strength of Oncogenic ras Signals**—Although \textit{ras} is frequently activated in human tumors, the expression levels of activated \textit{ras} vary from severalfold to more than 50-fold (6, 27). In fact, \textit{ras} genes activated through point mutations are usually not overexpressed at very high levels. This prompted us to explore the
relationship between the signaling intensity of oncogenic \textit{ras} and its ability to induce senescence in primary human cells.

Two retroviral expression vectors, both derived from the same Moloney murine leukemia virus-based expression plasmid (WZL-Hygro), were used to achieve differential expression levels of \textit{Ha-rasV12} (Fig. 1A). WZL-Hygro-HaRasV12 was used previously to express \textit{Ha-rasV12} and induce senescence (21, 24). A second binary vector was essentially the same as WZL-Hygro-HaRasV12 but contained an additional neutral gene (GFP or GAPDH) and a PGK promoter downstream of the 5'-long terminal repeat. In this vector, the expression of the upstream neutral gene was directed by the viral long terminal repeat, whereas the downstream \textit{Ha-rasV12} was expressed from the PGK promoter. We consistently found that expression of \textit{Ha-rasV12} was reduced by the presence of the upstream gene. By using these two vectors, \textit{Ha-rasV12} was stably transduced into early passaged human foreskin fibroblast cells (BJ). In the presence of an upstream gene (GFP, GAPDH, E6, or E6E7), the expression levels of Ha-RasV12 from the binary vectors were 3–6-fold higher than that in the control cells not transduced with \textit{ras} (Fig. 3). On the other hand, the WZL-Hygro-HaRasV12 vector conferred more than 20-fold overexpression of \textit{ras}. Ras overexpressed by more than 20-fold led to a higher degree of activation of its downstream signaling components, MEK and ERK, than when overexpressed by 3–6-fold (Fig. 3), indicating that higher expression levels of \textit{ras} had indeed generated stronger \textit{ras} signals in BJ cells.

Confirming previous findings, Ha-RasV12 induced premature senescence in BJ cells when overexpressed from WZL-Hygro at high levels (Fig. 1, B and C). These cells stopped proliferating and accumulated senescence-associated \textbeta-galactosidase, a marker distinguishing senescence from other types of growth arrest (36). In contrast, when Ha-RasV12 was expressed at relatively lower levels from the binary vectors, cells did not undergo premature senescence. Unlike the cells with high levels of \textit{ras}, these cells showed exponential growth kinetics (Fig. 1B) and did not accumulate senescence-associated \textbeta-galactosidase (Fig. 1C). In addition, these cells displayed normal morphology of fibroblasts, whereas cells expressing high \textit{ras} levels became large and flat, showing morphology typical of senescence (data not shown). BJ cells transduced with binary vectors expressing only GFP or GAPDH grew at similar rates as those transduced with the WH vector, indicating that GFP and GAPDH alone had no effect on cell proliferation. Thus, the ability of oncogenic \textit{ras} to induce senescence depends on the intensity of its expression levels. Oncogenic \textit{ras} provokes senescence-like growth arrest in primary human fibroblasts only when it is highly expressed.

\textbf{Oncogenic ras Is Mitogenic When Expressed at Moderate Levels}—Whereas the moderate \textit{ras} expression levels did not trigger senescence in BJ cells, we determined the ability of \textit{ras} to promote cell proliferation when expressed at moderate levels. BJ cells overexpressing Ha-RasV12 by 3–5-fold above the background from the binary vectors consistently displayed higher growth rates than those transduced with the vector control, when the cells were continuously grown in complete medium (Fig. 1B). We estimated that the doubling time of the cells reduced from 1.4 days to 1.0 day upon the transduction of
moderate expression levels of ras. To determine further the mitogenic activity of ras, the rate of BrdUrd incorporation was measured in BJ cells under serum starvation. Transduction of BJ cells with moderate expression levels of ras resulted in a 2–3-fold increase in BrdUrd incorporation when the cells were grown in a low serum-containing medium (Fig. 2). A similar degree of increase in BrdUrd incorporation was observed in cells transduced with high levels of ras, before premature senescence occurred (Fig. 2), confirming published observations (24, 26). Expression of GFP or GAPDH alone from the binary vectors had no effect on the rate of BrdUrd incorporation (Fig. 2), suggesting that the increases in BrdUrd incorporation observed in cells transduced with the binary vectors were indeed due to the moderate ras expression. Therefore, although ras signals of moderate intensity did not induce senescence, they stimulated proliferation in primary BJ human fibroblasts as did the high intensity ras signals. This notion was confirmed by the finding that both moderate and high intensity ras signals led to increased protein levels of cyclin E (Fig. 3), which plays an important role in promoting G1/S transition. Thus, our results demonstrated that ras triggered opposing cellular responses when present in cells at different intensity. Although moderate ras activity was mitogenic, higher ras activity led to growth arrest and premature senescence.

**Ras Signals of Different Intensity Differ in Their Abilities to Activate the ERK and p38 MAPK Pathways and to Induce Senescence-associated Changes**—One of the downstream effectors that ras relies on to transmit mitogenic signals and confer tumorigenesis is the Raf/MEK/ERK MAPK pathway (37, 38). This pathway is also essential for ras to induce the senescence response in primary cells (24, 25). We have showed earlier that active MEK leads to the activation of p38 MAPK that in turn mediates the induction of p16INK4A and senescence (26). In order to explore the possible molecular mechanism underlying the dependence of the cellular responses on the strength of ras expression, we examined whether ras, when expressed at different levels, differentially regulates the MAPK pathways that mediate growth stimulation and senescence response.

The high expression levels of Ha-RasV12, as well as the moderate levels of Ras that did not induce senescence, led to the activation of MEK and ERK through phosphorylation of their activating sites (Fig. 3). It is important to note that the extent of MEK and ERK activation, as revealed by the levels of these kinases phosphorylated at their activating sites, were proportional to the ras expression levels, demonstrating that the expression levels of ras correlated with its signaling strength. However, the activating phosphorylation of p38 was only induced by more than 20-fold overexpression of ras, but not by the moderate levels (3–5-fold overexpression) of ras (Fig. 3). The ERK and p38 pathways were not activated in control cells expressing GFP or GAPDH alone from the binary vectors (data not shown), suggesting that the observed changes in these MAPK pathways in cells transduced with the binary vectors resulted from moderate ras expression. Therefore, the specificity in the activation of different MAPK pathways is determined by the intensity of ras signaling. This finding has revealed a molecular mechanism by which different levels of ras activity can lead to opposing cellular responses. Whereas moderate intensity ras signals only activate the mitogenic MEK-ERK pathway and promote proliferation, high intensity ras signals induce the activation of MEK and ERK to higher levels, leading to the stimulation of p38 activity that in turn induces p16INK4A and triggers premature senescence.

Oncogenic ras-induced premature senescence is accompanied by elevated expression levels of p16INK4A and p21Cip1 and declines in the levels of hyperphosphorylated Rb and cyclin A proteins (21, 24, 25). These changes were consistent with the observation that senescent cells were arrested in G1 phase. We found that Ha-RasV12, when expressed at senescence-inducing levels (i.e. more than 20-fold overexpression), enhanced the protein levels of p16INK4A and p21Cip1 and reduced the levels of hyperphosphorylated Rb and cyclin A proteins (Fig. 3). In contrast, these changes were not provoked by the moderate levels (3–5-fold overexpression) of ras. These observations confirmed that ras signals of moderate intensity did not induce premature senescence in BJ human fibroblasts. In addition, both high and moderate levels of ras increased the expression of cyclin E in BJ cells (Fig. 3), in agreement with previous reports (39, 40) demonstrating the ability of ras to induce cyclin E in immortalized murine cells (NIH3T3). This finding suggested that oncogenic ras was able to stimulate the cell cycle machinery and promote cell proliferation even when overexpressed by only several fold above the basal levels. In contrast to the observation in NIH3T3 cells, cyclin D1 levels were not significantly increased.

**Fig. 2. Mitogenic activity of ras when expressed at moderate levels.** 3 days after retroviral infection, BJ cells (PD25) transduced with the WH vector, binary vectors expressing GFP or GAPDH alone, and binary vectors expressing Ha-RasV12 downstream of GFP, GAPDH, or WH-Ha-RasV12 were grown in medium containing 0.1% fetal calf serum for 24 h and labeled with 50 μM BrdUrd for 2 h. Cells were stained with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and propidium iodide and analyzed for BrdUrd incorporation and DNA content by 2-color flow cytometry. The box (R3) in each panel represents cells incorporating BrdUrd (i.e. cells in S phase). The percentage of BrdUrd-positive cells is indicated for each cell line.
by either high or moderate levels of ras in BJ primary human fibroblasts (Fig. 3).

**Inducible Expression of Ha-RasV12** — To confirm that the ability of ras to induce the p38 pathway and senescence relies on high signaling intensity, we analyzed BJ human fibroblast cells expressing Ha-RasV12 from a doxycycline-inducible promoter (33). In order to construct a cell line in which an exogenous gene can be uniformly induced, clonal cell populations need to be isolated and propagated. To achieve this in the mortal BJ cells, we took advantage of the fact that hTERT immortalizes cells but does not bypass ras-induced senescence (42, 43). BJ cells were immortalized with an hTERT-retrovirus, and then sequentially transduced with a virus expressing the tetracycline transactivator rtTA (33) and then a virus expressing Ha-RasV12 under the control of a doxycycline-inducible promoter. Clonal populations transduced with both viruses were selected and examined for ras inducibility by doxycycline.

In order to investigate the effect of ras signal intensity on the proliferative property of BJ fibroblasts, we performed analysis in one clone that displayed an incremental induction (from 2- to more than 10-fold over the basal level) of ras expression by increasing concentrations of doxycycline (Fig. 4C). Because up to 2 μg/ml doxycycline did not appear to be toxic to these cells, and had no effect on the proliferation or the MAPK pathway in wild-type BJ cells (data not shown), doxycycline concentrations ranging from 0.01 to 1 μg/ml were used in this experiment. When ras was induced to more than 10-fold above the basal level by 1 μg/ml doxycycline, it initially promoted cell growth (Fig. 4A). Within the first 3 days following treatment with 1 μg/ml doxycycline, these cells underwent 1.1 ± 0.1 population doublings, resulting in twice as many cells as compared with the control cells in which ras was not induced (Fig. 4A). However, ~7 days into the doxycycline treatment, cells with more than 10-fold ras induction started to exhibit a slower growth rate and eventually stopped proliferating completely (Fig. 4B). These arrested cells showed a senescent morphology and were positive for senescence-associated β-galactosidase activity (data not shown). BJ cells with 5–7-fold ras induction by 0.3 μg/ml of doxycycline also acquired a higher growth rate than the uninduced cells (Fig. 4, A and B). In contrast to the cells expressing higher levels of ras, these cells with 5–7-fold ras induction did not undergo premature senescence but continued to proliferate at a rate higher than that of the uninduced control cells (Fig. 4, A and B). Doxycycline at 0.1 μg/ml and below induced very little ras expression (Fig. 4C) and did not significantly alter the growth property of BJ cells (Fig. 4, A and B). These findings have clearly demonstrated that the ability of ras to induce senescence is determined by its expression levels. Oncogenic ras can stimulate cell proliferation without triggering premature senescence when the expression is induced by severalfold. Higher expression levels of ras are initially mitogenic but eventually lead to senescence when the overexpression is sustained.

We further analyzed the effects of differential ras induction levels on the activation of the MEK-ERK and MKK3/6-p38 pathways in the BJ clone expressing inducible Ha-RasV12 (Fig. 4C). The activating phosphorylation of MEK and ERK was induced moderately by 5–7-fold overexpression of ras and to higher levels by more than 10-fold overexpression of ras. On the contrary, the activating phosphorylation of MKK3/6 and p38 only occurred in cells with more than 10-fold ras induction and was not observed when ras expression was induced to a few-fold above the basal level (Fig. 4C). In agreement with the observation that only high levels of ras induction led to the stimulation of the p38 pathway and premature senescence, p16INK4A was only significantly induced in cells with more than 10-fold overexpression of ras (Fig. 4C, day 10 panel). These results confirmed our finding using retroviral vectors that mediated differential ras expression levels, demonstrating that the senescence-inducing p38 pathway was stimulated only by high intensity ras signals, whereas the mitogenic MEK-ERK pathway could be activated even when ras was expressed at moderate levels. Moreover, the two MAPK pathways also displayed distinct kinetics of activation by ras (Fig. 5C). The activation of MEK and ERK occurred immediately after ras induction and was readily detectable on day 1 following the initiation of doxycycline treatment. Activation of ERK seemed to be slightly delayed as compared with that of MEK, with the maximal levels reached on day 5. In contrast, the MKK3/6-p38 pathway was activated by ras much later than the MEK-ERK pathway. The increased phosphorylation of MKK3 and p38 was apparent on day 10 after the initial ras induction, suggesting that the activation of these kinases occurred between day 5 and day 10. The induction of p16INK4A followed the same pattern as that of the p38 pathway, in that the expression level of p16INK4A was only significantly elevated between day 5 and day 10 following more than 10-fold induction of ras. The delayed induction of MKK3/6 and p38 suggested that the senescence-inducing p38 pathway was activated as a result of the sustained presence of high intensity Ras-MEK-ERK signaling, and that MEK stimulated MKK3/6 and p38 probably through an indirect mechanism.

Taken together with our previous finding that the p38 pathway acts downstream of the Ras-MEK cascade to mediate premature senescence, the results from the BJ cells expressing inducible ras have indicated that the ability of ras to induce distinct cellular responses can be attributed to the fact that ras signals of different intensities are able to differentially regulate the MAPK pathways that mediate these responses. Moderate expression levels of ras initiate moderate intensity ras signaling and induce the MEK-ERK pathway to a level that is sufficient to promote cell proliferation but not to trigger the activation of the MKK3/6-p38 pathway or premature senescence. Stronger ras signals generated by high levels of ras expression stimulate the activity of the MEK-ERK pathway to a higher
Fig. 4. Analysis of the growth property and MAPK activation in a BJ cell clone that displays incremental induction of Ha-RasV12 by increasing concentrations of doxycycline. A, total number of cells obtained after the first 3 days of growth in the presence of indicated concentrations of doxycycline. 10^4 cells were seeded initially when the treatment started. B, growth curves of the BJ clone expressing inducible Ha-RasV12 in the presence of indicated concentrations of doxycycline (Doxy). Numbers of cells over a 14-day period are shown. Values are means ± S.D. for duplicates. C, Western blot analysis of the BJ clone expressing inducible Ha-RasV12 after 1, 5, and 10 days of treatment with the indicated concentrations (μg/ml) of doxycycline, showing different induction levels of Ha-RasV12 and protein levels of activated MEK, activated ERK, ERK2, activated MKK3/6, MKK3, activated p38, p38, and p16<sup>INK4A</sup>. The ECL signals were captured by the FluorChem™-8900 Imaging System, and the relative levels of Ras protein and activated ERK were quantified by the FluorChem™-8900 software, normalized to the basal levels in the untreated cells and indicated in the figure.

Transformation of Primary Human Fibroblasts by Moderate Levels of ras—Because ras expression levels vary among human tumors, we investigated whether the moderate intensity of ras signals that promoted proliferation, but did not induce senescence, could also provide sufficient oncogenic stimuli for the transformation of primary human cells. In several tumor cell line models that were established previously, oncogenic ras cooperated with other oncogenes in the transformation of primary human and rodent cells (3–6, 44). In particular, studies on primary human cells have indicated that in addition to ras, transformation requires that these cells be immortalized (by hTERT), and that both Rb and p53 pathways be inactivated (by SV40 LT, HPV-16 E6E7 or other elements) (5, 6). We examined whether moderate expression levels of activated ras can transform immortalized BJ fibroblasts in cooperation with HPV-16 E6 and E7 oncogenes that target p53 and Rb, respectively.

E6E7 and Ha-RasV12 were transduced via the binary retroviral vector (Fig. 1A) into BJ human fibroblasts that had been immortalized by hTERT. Consistent with previous reports (45–47), E6 and E7 led to nearly complete degradation of p53 and Rb proteins in BJ cells (Fig. 5B). Ha-rasV12 was placed downstream in the binary vector, to achieve moderate expression levels (3–5-fold above the control level) (Fig. 3) that were not sufficient to induce prematurity senescence (Fig. 1C and data not shown). Expression of hTERT, E6E7, and moderate level of ras in BJ cells promoted anchorage-independent growth on soft agar (Table 1). When injected subcutaneously into athymic nude mice, these cells readily formed tumors (Table I and Fig. 5A). The tumors became visible about 35 days after injection and started quick growth afterward (Fig. 5A). Hematoxylin and eosin staining showed that the tumor cells were poorly differentiated, with small cytoplasm and large nuclei, and that these tumors were invasive (data not shown). However, although more careful studies are required to determine the metastatic potential of these tumors, no obvious metastases were observed in lung, liver, spleen, and kidney. As a control, hTERT and E6E7 alone did not transform BJ cells. Therefore, moderate intensity of ras signals was both necessary and sufficient to confer tumorigenicity to primary human fibroblasts in cooperation with E6E7 and hTERT. This finding implies that the ability of ras to promote proliferation and oncogenic transformation can be uncoupled with its ability to induce senescence in cell culture. In BJ cells transformed by hTERT, E6E7, and moderate strength of Ha-RasV12, the level of activated p38 was unaltered as compared with that in the control cells, whereas the level of activated ERK was increased. The same pattern of MAPK activation was observed in cells recovered from the tumors (Fig. 5B), suggesting that the p38 pathway might not contribute to ras-mediated transformation in BJ cells in the presence of E6E7 and hTERT.

Transformation of BJ cells was also achieved with the same elements expressed in a different configuration. In this case, E6 alone was expressed upstream of Ha-RasV12, and E7 was expressed from a separate vector. We noticed a 2-fold increase in the expression level of Ha-RasV12 when it was placed down-
stream of the E6 gene than when it was downstream of the entire E6/E7 region (Fig. 3). Interestingly, the increased Ha-RasV12 expression level from the E6-PGKRas vector did not induce senescence (data not shown) but seemed to enhance tumorigenicity of the transformed BJ cells. BJ-hTERT-E6E7-PGKRas cells formed significantly more soft agar colonies than BJ-hTERT-LXSN-E6E7-PGKRas cells (Fig. 3), indicating that the parental cell line and the transformed BJ cell line were both polyclonal in nature. Therefore, the transformed BJ cell line was not established from a small number of cells carrying rare secondary mutations. Tumor 2 contained less retroviral insertions, suggesting a clonal selection might have occurred during the growth of this tumor. However, tumor 2 was allowed to grow to a larger size (>5000 mm³) before removal, and as a result, only a small portion of cells was adapted into the culture. Thus, it is equally possible that the number of viral integrations in this tumor might have been under-represented in this assay.

It was reported that transformation of human mammary epithelial cells by LT, hTERT, and ras might have been facilitated by additional changes (e.g. c-myc amplification) that were apparent at the karyotypic level, whereas transformation of kidney epithelial and fibroblast (BJ) cells by the same elements did not involve these changes (6). To investigate whether additional karyotypic alterations were involved in our transformation model, karyotype analysis was performed on pre- and post-tumor cells expressing hTERT, E6, E7, and moderate levels of ras (Table II). All the cells examined were diploid. Abnormal karyotypes were only observed randomly in a small percentage of cells in certain samples. 5% of the cells transformed by hTERT and E6E7-PGKRas had abnormal 10q before injection, but all of them were normal when explanted from the tumors. Cells transduced with hTERT, LXSN-E7, and E6-PGKRas were all normal before injection, and abnormalities were detected in a few cells in the explanted populations. One tumor contained 5% of cells with an abnormal 10q and 5% with loss of tumor 11, and the other tumor had 5% of cells with rearrangements on tumor 3, 5% with an addition on 3p, and 5% with multiple translocations with unknown origins involving 3p. The fact that the majority of the pre- and post-tumor cells had a normal karyotype indicated that no additional alterations had occurred at the chromosomal level during transformation of primary human fibroblasts by moderate expression levels of ras.
The Giemsa-stained soft agar colonies were scored 4 weeks after $10^6$ cells had been plated into 6-well plates. Values are means ± S.D. for triplicate experiments. Tumor formation in nude mice was monitored within a period of 3 months after $5 \times 10^6$ cells had been injected subcutaneously into athymic WEHI nude mice.

| Genotype                  | No. soft agar colonies/10⁴ cells plated | No. tumors injections |
|---------------------------|----------------------------------------|-----------------------|
| BJ-hTERT, E6E7-PGKHaRasV12| 146 ± 11                               | 9/12                  |
| BJ-hTERT, E6E72Pro-PGK HaRasV12 | 23 ± 6                                | 0/6                   |
| BJ-hTERT, E6E72Gly-PGK HaRasV12 | 18 ± 9                               | 0/6                   |
| BJ-hTERT, E7-PGK HaRasV12   | 21 ± 4                                 | 0/6                   |
| BJ-E6E7-PGKHaRasV12          | 82 ± 21 (sm)                           | 0/6                   |
| BJ-hTERT, E6E7               | 15 ± 4                                 | 0/4                   |
| BJ-hTERT, E7, E6-PGKHaRasV12 | 192 ± 26                              | 3/3                   |

All together, these results indicate that the transformation of primary human fibroblasts by moderately expressed oncogenic ras, hTERT, and E6E7 may not require additional, rare genetic changes besides the transduced oncoproteins. Although these analyses did not rule out the involvement of secondary mutations that occurred at high frequency and were undetectable at the karyotypic level, our data clearly demonstrated that moderate intensity of ras signals was sufficient to promote oncogenic transformation, and that activation of the p38 pathway might only serve to mediate the senescence response in primary fibroblasts and did not contribute to the oncogenic activity of ras.

**DISCUSSION**

In this study, we have investigated the relationship between the biological consequences of ras activation and the signaling intensity of activated ras in primary human fibroblasts. Our results have demonstrated that the biological outcome of ras activation in primary human cells is modulated by the strength of ras signals. A moderate (severalfold) increase in oncogenic ras expression causes activation of the mitogenic MEK-ERK MAPK pathway and stimulation of cell growth, without inducing the MKK3/6-p38 pathway or senescence response. Further elevation of ras expression (to more than 10-fold above the basal level) stimulates the activity of MEK and ERK to even higher levels and initially promotes proliferation, but the sustained presence of a stronger ras signal eventually leads to activation of the MKK3/6-p38 pathway and induction of p16INK4A and triggers premature senescence. We have shown previously (26) that MKK3/6 and p38 act downstream of MEK to mediate ras-induced senescence and that the ability of ras to induce senescence relies on an active p38 pathway. Thus, our data have revealed a molecular mechanism for the differential effects of ras on cell proliferation when expressed at different levels. Moderate expression levels of ras expression promote proliferation but fail to induce senescence, because they provide cells with moderate intensity ras signals that are sufficient to activate the MEK-ERK pathway but are not strong enough to stimulate the p38 activity. In contrast, stronger ras signals induce MEK-ERK to higher levels that trigger the activation of the p38 pathway and senescence. The dependence of senescence response on high intensity ras signaling also mirrors two previous observations (39, 40) in immortalized NIH3T3 murine fibroblasts, where oncogenic Ras and its downstream effector Raf caused G1 growth arrest only when expressed at high levels. A similar difference in the ability to activate the p38 pathway may also account for the differential capability of Ras in inducing G1 arrest when expressed at different levels in NIH3T3 cells.

Two experimental approaches were used during the course of this study, one utilizes separate (but similar) retroviral vectors that drive different levels of ras gene expression, and the other allows incremental induction of ras expression in a same cell population in response to doxycycline treatment. Each one of these two approaches complemented the shortcomings of the other in demonstrating that the cellular outcomes of ras activation depended on the ras signaling intensity. All the vectors used in the first approach were derived from a same parental retroviral vector (WZL-hygro), with the only exception of the promoters driving ras expression and the neutral genes such as GFP or GAPDH present upstream of ras. Although the presence of GFP or GAPDH and the PGK promoter appeared to be neutral to the phenotypes investigated in this study in that they had no effect on cell proliferation and MAPK activation, we cannot exclude the unlikely possibility that these neutral elements may somehow affect the cells in an unexpected and undetectable way. The doxycycline-inducible system allowed us to follow the effects of different ras levels in a real time fashion in a single cell clone containing cells with an identical genetic background, thus avoiding the possible complications of using different expression plasmids. However, clonal variations are inevitable in mammalian cells and sometimes may complicate the results derived from a small number of single-cell clones. Moreover, doxycycline may also inadvertently interfere with certain cellular behaviors. In contrast, the first approach using retroviral expression vectors permits the analysis of ras effects in cell populations, rather than in a few single-cell clones, without doxycycline. In this study, results obtained with these two different approaches led to the same conclusions.

We have demonstrated in a previous report that MKK3/6 and p38 activities are stimulated as a result of sustained activation of MEK by ras (26). Results from this study have confirmed this conclusion. In BJ cells expressing inducible ras, activated MEK and ERK were detectable after 1 day following the initial ras induction, whereas activated MKK3/6 and p38 did not accumulate significantly until after day 5. The delayed activation of MKK3/6 and p38 by ras suggests that MEK does not activate MKK3/6-p38 through direct signaling. Rather, multiple steps may be engaged in mediating the activation of MKK3/6-p38 by MEK in response to ras induction. Some of these steps may involve slow mechanisms such as transcriptional regulation and others that require de novo protein synthesis. At present, the role of ERK in the activation of MKK3/6-p38 and induction of senescence remains unclear. The timing of MKK3/6 and p38 activation also correlated with the appearance of senescence, which occurred between day 7 and day 10 following ras induction (Fig. 4, B and C). This observation has offered an explanation to why premature senescence is a late response to ras activation in normal cells (24), and we have confirmed the role of p38 pathway in mediating this response.
Although moderate ras activity did not trigger premature senescence, it provided cells with sufficient mitogenic signals that stimulated cell proliferation and generated adequate oncogenic activities that led to cellular transformation in cooperation with E6E7 and hTERT. Therefore, the ability of oncogenic ras to promote cell proliferation and oncogenic transformation can be uncoupled with its ability to induce premature senescence in cell culture. The transformation was accompanied by activation of the ERK pathway but occurred without stimulation of the p38 pathway (Fig. 5B), suggesting that p38 may not be required for the oncogenic activity of ras. Therefore, the role of p38 in the ras pathway may be limited to mediating the senescence response that serves as an anti-oncogenic defense mechanism in normal cells. It has been well documented that ras-induced senescence is an important barrier for transformation in both rodent and human cells, and that transformation by ras needs additional alterations that allow the bypass of this barrier (21, 24, 25). Because human tumors express varied levels of ras, our studies suggest that cells with different levels of oncogenic ras may have different requirements for transformation. Although the formation of tumors with high ras expression requires additional genetic alterations that overcome the senescence response, these additional changes may not be essential in the development of tumors where ras is expressed at moderate levels.

There has been an ongoing effort to create tumor cell line models by introducing defined genetic elements into normal primary cells. These models would provide ideal systems for analyzing the genetic requirements for oncogenic transformation. Several papers reported the creation of transformed human tumor cell lines using hTERT, oncogenic ras (expressed at high levels), and SV40 LT or HPV16 E6E7 (5, 6, 29). SV40 small t antigen (st) might also be required in this transformation model (29). In primary human mammary epithelial cells, st seemed to contribute to transformation by activating the phosphatidylinositol 3-kinase pathway (51). The requirement of SV40 st was consistent with a previous observation that hTERT, E6E7, and Ha-RasV12 alone did not transform human cells (52). Here we have successfully transformed primary human fibroblasts with hTERT, E6E7, and moderate expression levels of Ha-RasV12, without SV40 st. The analysis of the transformed cells indicated that transformation did not involve the selection of additional, random genetic alterations other than the transduced oncogenes. In contrast, hTERT, E6E7, and high ras levels did not lead to transformation of BJ cells in the absence of st. Although other possibilities cannot be ruled out, we speculate that the different requirement for st may result from the differential ras expression levels. The ras expression vector (Babe-puro) used in the prior studies conferred high expression levels. SV40 st might be needed to overcome certain growth restraints induced by high levels of ras either in cell culture or in mice. By lowering the ras signaling strength, we might have relieved these barriers created by high expression levels of ras, and thus eradicated the requirement of st. In addition, BJ cells expression of hTERT, E6E7, and oncogenic ras of moderate intensity seemed to develop tumors in nude mice at a slower rate than those transformed by hTERT, SV40 LT region (including st), and highly expressed oncogenic ras in the models reported previously (5). The difference could again be due to different ras levels used in these studies. Higher expression levels of ras may provide cells with stronger mitogenic signals leading to more efficient transformation, especially if the negative growth impact of high intensity ras signals has been bypassed. Indeed, a 2-fold increase in the ras level led to enhanced tumorigenesis in our study. Alternatively, the presence of st may facilitate transformation, regardless of its role in overcoming the negative effect of ras on proliferation. Despite the differences in the requirement of SV40 st and the rate of tumor growth, we have clearly demonstrated in our model that moderate ras activity is sufficient to confer oncogenic transformation in primary human fibroblasts, in cooperation with hTERT and E6E7.

High risk HPVs are associated with more than 90% of human cervical cancer (53). E6 and E7 are the major transforming proteins of HPV-16 (54, 55). However, HPV alone is not sufficient to cause tumorigenesis, suggesting the involvement of other cellular changes (56, 57). Coincidentally, oncogenic ras mutant alleles were found in as many as 40% of human cervical cancer (41, 58). Therefore, our transformed cell line model is highly relevant to human cervical cancer. In this model, the complete transformation requires cell immortalization by hTERT, oncogenic signals provided by ras, and inactivation of p53 and Rb pathways by E6 and E7, respectively. Most significantly, oncogenic ras confers tumorigenic potential when expressed at levels that are not sufficient to cause premature senescence in cell culture. Therefore, if this model can be extended to human cervical epithelial cells, our findings would suggest that some of the genetic mutations that are responsible for bypassing ras-induced senescence might not be required in at least a subset of human cervical tumors with moderate ras activity.

Acknowledgments—We thank Drs. R. Weinberg, S. Lowe, D. Beach, J. Smith, S. Reed, J. Wang, R. Maestro, and D. Galloway for providing reagents; Dr. P. Vogt for critical review of the manuscript; and Angela Hatch for administrative assistance.

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Karyotype analysis was performed in transformed cells before they were injected into mice (pre-tumor) and after explanted from subcutaneous tumors (tumor). % of cells with a normal karyotype (46, XY) or with karyotypes of indicated abnormality is shown within each sample.

| Genotype of BJ | Cells                  | Karyotype                        |
|---------------|------------------------|----------------------------------|
| hTERT, E6E7-PGKras | Pre-tumor              | 100% 46, XY, 5% (?10q)           |
|               | Tumor                  | 100% 46, XY                      |
| hTERT, E7, E6-PGKras | Pre-tumor              | 100% 46, XY, 5% (?10q; 5% del(11)) |
|               | Tumor 1                | 100% 46, XY, 5% der(3); 5% add(3p); 5% (?)(p)(?)(?)(?) |
|               | Tumor 2                | 100% 46, XY, 5% del(3); 5% add(3p); 5% (?)(p)(?)(?)(?) |
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