Platelet-derived Growth Factor and Reactive Oxygen Species (ROS) Regulate Ras Protein Levels in Primary Human Fibroblasts via ERK1/2

AMPLIFICATION OF ROS AND Ras IN SYSTEMIC SCLEROSIS FIBROBLASTS

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The levels of Ras proteins in human primary fibroblasts are regulated by PDGF (platelet-derived growth factor). PDGF-induced post-transcriptionally Ha-Ras by stimulating reactive oxygen species (ROS) and ERK1/2. Activation of ERK1/2 and high ROS levels stabilize Ha-Ras protein, by inhibiting proteasomal degradation. We found a remarkable example in vivo of amplification of this circuitry in fibroblasts derived from systemic sclerosis (sclerodermia) lesions, producing vast excess of ROS and undergoing rapid senescence. High ROS, Ha-Ras, and active ERK1/2 stimulated collagen synthesis, DNA damage, and accelerated senescence. Conversely ROS or Ras inhibition interrupted the signaling cascade and restored the normal phenotype. We conclude that in primary fibroblasts stabilization of Ras protein by ROS and ERK1/2 amplifies the response of the cells to growth factors and in systemic sclerosis represents a critical factor in the onset and progression of the disease.

Although the detailed molecular nature of the link between oncogenesis and senescence remains obscure, they appear to be two sides of the same coin. Ras and reactive oxygen species (ROS) are two important players that underlie both phenotypes (transformation and senescence), but their effects are somewhat enigmatic. For example, in mammalian cells, expression in fibroblasts of the oncogenic allele of ras (v-Ha-Ras) triggers rapid senescence (1). Also, ROS mediate apoptosis, DNA damage (2), RNA synthesis (3), as well as growth inhibition (4).

ROS and Ras signaling are linked in the yeast Saccharomyces cerevisiae. cAMP-PKA signals are located downstream of Ras. However, constitutively active Ras2Val19 affects endogenous ROS production and oxygen consumption in a PKA-independent way (5).

The on-line version of this article (available at http://www.jbc.org) contains supplementary material.

1 The abbreviations used are: ROS, reactive oxygen species; PKA, cAMP-dependent protein kinase; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; GST, glutathione S-transferase; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; NAC, N-acetyl cysteine; ERK, extracellular signal-regulated kinase; DPI, diphenylene iodonium.

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1 To whom correspondence may be addressed: Dept. Biol. Pat. Mol. Cell. Via S, Pansini 5, 80131 Napoli, Italy. Tel.: 39-81-746-3251; Fax: 39-81-746-3252; E-mail: avvedim@unina.it.

2 To whom correspondence may be addressed: Ist. Clinica Medica, via Trento, 10, 60020, Ancona, Italy. Tel.: 39-71-2206104; Fax: 39-71-2206103; E-mail: a.gabrielli@unipv.it.

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(F234), anti-pan-Ras (F132), and anti-Rac1 antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-p44/42 MAP kinase, anti-phospho-SAPK/JNK and anti-AKT from Cell Signaling Technology (Beverly, MA). Anti-H2AX and anti-p21WAF antibodies were obtained from Upstate Biotechnology (Charlottesville, VA); diphenylene iodonium (DPI) from Alexis Biomedicals (Lansen, CH), N-acetyl-l-cysteine (NAC), and cycloheximide from Sigma. U0126 from Promega (Madison, WI) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes (Eugene, OR). The following plasmids were employed: dominant-negative Ha-rasN17, V12 positive variant of human Ha-ras and V12 positive variant of human Ki-Ras (7), dominant-negative Rac variant (Rac1N17), dominant-positive Rac variant (Rac1V12) (13), dominant-negative MEK variant pBabe-MKK2 (18) (rat GenBank™, X02163). The cDNA for collagen α1(I) (H1677 clone) and for collagen α2(I) (H302 clone) were kindly donated by Dr. Ch. M. Lapiere (Laboratoire de Biologie des Tissues Conjonctifs, University of Liege, Belgium).

Primary Fibroblasts—Human skin fibroblasts were obtained from punch biopsies taken from the forearms of normal volunteers and from the involved skin of patients who fulfilled the preliminary criteria of the American Rheumatism Association for the diagnosis of systemic sclerosis as described (14). Fibroblasts between the fourth and the sixth subpassage were used for all experiments.

Flow Cytometric Analysis with Anti-Ha-Ras Antibody—Cells were grown to semiconfluency in 60-mm culture dishes. After trypsin detachment, 5 × 10⁵ cells were suspended in 1 ml of phosphate-buffered saline (PBS) and fixed overnight with 1% formaldehyde at room temperature. Next, cells were permeabilized with 0.1% Triton X-100 for 40 min at 4 °C, washed four times with 2 ml of PBS containing 2% FCS, 0.01% NaN₃, 0.1% Triton X-100 (buffer A), and incubated for 45 min at 4 °C with 1:50 dilution of monoclonal and polyclonal anti-Ha-Ras antibodies (Santa Cruz Biotechnology). The cells were then washed twice with the same buffer and incubated for 45 min at 4 °C with Cy2-conjugated anti-mouse IgG antibodies (Amersham Biosciences) at 1:50 dilution. Control cells were incubated with Cy2-conjugated anti-mouse IgG antibodies alone. After two washes in buffer A, cells were resuspended in PBS and analyzed by flow cytometry using FACScan (BD, Heidelberg, Germany) and WinMDI software.

ROS Determination—Fluorometric determination of intracellular ROS generated by fibroblasts was estimated after loading the cells with DCFH-DA (10 μM) for 40 min at 37 °C before assessing DCF fluorescence level (15). Superoxide anion release was estimated using the superoxide dismutase-inhibitable cytochrome c reduction (12). H₂O₂ release from fibroblasts into the overlying medium was assayed using a modification of the method of Valletta and Berton (16). Oxidative activity imaging in living, transfected cells was evaluated as described (12, 17).

Immunoblotting—Cell culture plates were lysed with 0.3 ml of cold radioimmune precipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM sodium orthovanadate, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and processed as described (14).

Ras Immunoprecipitation—Ras proteins were immunoprecipitated from cultured fibroblasts with polyclonal anti-pan Ras antibody (Santa Cruz Biotechnology) following recommended procedures from the manufacturer. Immunocomplexes were isolated, electrophoresed, immunoblotted with anti-Ha-Ras or -Ki-Ras antibodies, and revealed by chemiluminescence (Amersham Biosciences).

Immunofluorescence—Fibroblasts, cultured on Lab-Tek chamber glass slides (Nalge-Nunc) and starved 48 h before stimulation or addition of inhibitors, were fixed, permeabilized, and stained with a monoclonal antibody against Ha-Ras or Ki-Ras and then with a tetramethylrhodamine isothiocyanate- (TRITC; Molecular Probes, PoortGeul, The Netherlands) conjugated secondary antibody. Slides were mounted with Vectashield (H-100; Vector, Burlingame, CA) and examined by fluorescence microscope. Acquired images were analyzed using the Laser Sharp Processing Bio-Rad software (version 3.2). All images from different slides condition were acquired in a blinded fashion.

Ras Activation Assay—Cells were washed in ice-cold PBS and lysed with 0.5 ml per plate of lysis buffer (20 mM Hepes, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lysates were cleared by centrifugation (13,000 rpm at 4 °C) and diluted to 1 mg/ml with lysis buffer. GST-RBD expression in transformed _Escherichia coli_ was induced with 1 mM isopropyl-1-thio-β-D-galacto-pyranoside for 1–2 h, and fusion protein was purified on glutathione-Sepharose beads. The beads were washed in a solution containing 20 mM Hepes, pH 7.4, 120 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 2 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. For affinity precipitation, lysates were incubated with GST-RBD prebound to glutathione-Sepharose (30 ml of packed beads) for 60 min at 4 °C with rocking. Bound proteins were eluted with SDS-PAGE sample buffer, resolved on 12% acrylamide gels and subjected to Western blotting. Blots were probed with anti-Ras, clone Ras10 (Upstate Biotechnology).

Transfection—For transfection experiments, confluent fibroblasts were plated in 100-mm dishes in culture medium. After 24 h, the medium was discarded, replaced with fresh culture medium, and the cells transfected. Transfection experiments were carried out in duplicate using a liposomal method (Effectene, Qiagen, Hilden, Germany).

In selected experiments the recombinant plasmid pGbClA2-P obtained by cloning the promoter of human type I collagen α2 chain gene (COL1A2) (20) was co-transfected with either pS3CAT carrying human catalase gene (a kind gift of Dr. Irani, The Johns Hopkins, Baltimore) or a control vector following the procedure described above. The luciferase activities of the samples were measured with a TD-20/20 luminometer (Turner Design) and the ratio of Renilla to firefly luciferase values was used to normalize the co-transfection experiments.

Apoptosis Assay—Normal and scleroderma fibroblasts were grown to 80–90% confluence and treated for 2 h with different concentrations of H₂O₂. When needed, cells were preincubated 30 min with PD 98059 (40 μmol/liter). Six hours after the removal of the stimulus, apoptosis was detected by FACS analysis using annexin V–Cy3 (Clontech, Palo Alto, CA).

RNA Isolation and Northern Analysis—Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen). Ten micrograms total RNA was then used for Northern blot analysis following a described procedure (12).

RT-PCR of Ha- and Ki-Ras mRNA—Total RNA and cDNA synthesis was performed as described (18). 2 μl of cDNA products (derived from 2.5 μg of total RNA) were amplified with 1 unit of Ampli Taq Gold (PE Applied Biosystems) in the buffer provided by the manufacturer, which contains no MgCl₂, and in the presence of the specific primers for Ha-, Ki-ras and actin genes (see below). The amount of dNTPs carried over from the reverse transcription reaction is fully sufficient for further amplification. Reactions were carried out in the GeneAmp PCR system 9600. A first cycle of 10 min at 95 °C, 45 s at 65 °C, and 1 min at 72 °C was followed by 45 s at 95 °C, 45 s at 65 °C, and 1 min at 72 °C for 30 cycles. The conditions were chosen so that none of the cDNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification, and that the two sets of primers were sufficient for the reaction and that the total amount of cDNA in the reaction was constant.

Under these conditions, we could observe a 2.5-fold increase in the expression of both Ha- and Ki-Ras mRNA in normal fibroblasts and a significant reduction of both Ha- and Ki-Ras mRNA in normal fibroblasts in the presence of Ha-Ras antibodies. The results were consistent with the assumption that the described method is suitable for the detection of low levels of ras mRNA in normal fibroblasts.
ers used in each reaction did not compete with each other. Each set of reactions always included a no-sample negative control. We usually performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. The following primers were used: Ki-
ras long, left primer: acatctctttgctgcccaat; right primer: gagc-
gagactctgacaccaa. Ki-
ras short, left primer: tcgacacagcaggtcaagag; right primer: aggcatcatcaacaccctgt. Ha-
ras, left primer: ccagctgatccagaaccatt; right primer: aggtctcgatgtaggggatg.

Cytogenetic Analysis—Cytogenetic studies were performed on fibro-
blasts before and after a 24-h incubation with FTI-277 (20 µM). Fibro-
blasts were cultured on cover glasses placed in a 35-mm Petri dish. After
adding 2 ml of Chang Medium® B the dishes were incubated for 48 h in
a 5% CO₂ incubator at 37 °C and the cultures harvested after adding
Colcemid solution (0.1 µg/ml) for 90 min. The cover glasses were fixed and Q-banded following standard procedures. The evaluation was per-
fomed using a fluorescence microscope Zeiss Axioplan 2. The images
were captured with a couple-charged camera device connected to a
personal computer running MacKtype 5.4 software (Powergene Olym-
pus Italy). Chromosome identification and karyotype designation were
made following the criteria of the International System for Human
Cytogenetic Nomenclature (ISCN).

Chromatin Extraction and Immunoblot for Phosphorylated H2AX—Cell culture plates were lysed with 0.2 ml of buffer (120 mM
NaCl, 40 mM Hepes, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM EDTA, 0.6%
Triton X-100, 2 mM sodium orthovanadate, 2 µg/ml aprotinin, 1 mM
phenylmethylsulfonyl fluoride) and centrifuged at 14,000 × g for 15 min.
A representative of three experiments is shown. E, semiquantitative RT-PCR of RNA extracted from cells stimulated with 15 ng of PDGF
for 60 min. The reactions were carried out as described under "Materials and Methods." Here a representative of three reactions (20 cycles) is shown. Under these conditions the intensity of the specific bands was linearly dependent on the concentration of cDNA. Ki long and short represent the two major Ki4B mRNAs differing in the length of 3'-untranslated region.

Statistical Analysis—Data are expressed as mean ± 1 S.D. Mean val-
ues were compared using Student’s paired and unpaired t test. p values
less than 0.05 were considered significant. All values are two-tailed.

RESULTS

Induction of Ras Protein Levels by PDGF—In quiescent primary
human fibroblasts Ha-Ras protein was almost undetectable. To deter-
Regulation of Ras Protein Levels in Primary Cells

The data presented above indicate that PDGF and ROS, when co-administered, induced Ha-Ras in primary fibroblasts. To investigate the mechanism and the link between PDGF and ROS, we employed PDGF and ROS scavengers, NAC (N-acetyl cysteine) and DPI (a specific inhibitor of NADPH oxidase). NAC and DPI both prevented the increase in Ha-Ras levels induced by PDGF, suggesting a role for ROS in the induction of Ha-Ras by PDGF.

FIGURE 2. ROS induce Ha-Ras in primary fibroblasts. A, time course of ROS induction by PDGF. Primary fibroblasts were plated and incubated in 0.2% FCS for 24 h, pretreated with NAC (20 mM) and DPI (20 μM) for 2 h, before the treatment with PDGF (15 ng/ml). Values represent mean ± S.D. of five independent experiments performed in duplicate. B, ROS inhibition abolishes induction of Ha-Ras by PDGF. Primary fibroblasts were treated with NAC (20 μM for 1 h) or DPI (20 μM for 1 h). C, fluorescence microscopy of fibroblasts preincubated with NAC and DPI. D, ROS mediate PDGF induction of ERK1/2. Primary fibroblasts were treated with NAC (20 μM) and DPI (20 μM) for 1 h, before the treatment with PDGF (15 ng/ml). E, ROS mediate PDGF induction of ERK1/2. Primary fibroblasts were treated with NAC and DPI, before the treatment with PDGF (15 ng/ml). Values represent mean ± S.D. of five independent experiments performed in duplicate.

PDGF Stimulation of ROS and ERK1/2—PDGF activates ROS production by stimulating NADPH oxidase (3, 19). The kinetics of ROS production following PDGF stimulation replicated that of Ha-Ras induction (Figs. 1 and 2A). To determine if ROS were involved in Ha-Ras induction by PDGF, we treated the cells with a nonspecific ROS scavenger (NAC) or NADPH oxidase inhibitor (DPI) and measured Ha-Ras levels in the presence or absence of PDGF. Fig. 2B shows that NAC and DPI significantly inhibited PDGF induced Ha-Ras levels. This is also shown by immunofluorescence with anti-Ha-Ras antibodies (Fig. 2C). The kinetics of Ha-Ras induction by PDGF in normal cells replicated also ERK1/2 activation profile (data not shown), suggesting a relation between MEK-ERK1/2 and Ha-Ras rise. To get an insight into this process, we measured Ha-Ras in cells pretreated with a chemical inhibitor of ERK1/2 signaling (PD 98059), which inhibits MEK (MAPKK), a kinase located upstream of ERK1/2 (MAPK). Treatment of the cells with PD98059 inhibited Ha-Ras induction by PDGF (Fig. 2D). In the same extracts, subjected to immunoprecipitation with anti-Ras antibodies, we measured PDGF receptor. Fig. 2D shows that PDGF treatment down-regulated the receptor, independently on ERK1/2 activation (Fig. 2D). To determine if ERK1/2 activation by PDGF was also sensitive to ROS depletion, we treated the cells with PDGF in the presence of a general ROS scavenger (NAC) or the NADPH oxidase inhibitor (DPI). Fig. 2E shows that ERK1/2 activated by PDGF was sensitive to NAC and DPI, indicating that ROS depletion interfered with ERK1/2 activation by PDGF. However, we noticed that a fraction of ERK1/2 induced by PDGF was resistant to NAC or DPI (Fig. 2E).

ROS Induce Ha-Ras Level—the data presented above indicate that PDGF via OS and ERK1/2 induce Ha-Ras protein levels in primary fibroblasts. To investigate the mechanism and the link between PDGF and ROS, we stimulated the cells with H2O2, in the presence of chemical and biological inhibitors of ERK1/2 signaling. To this end, we employed 1) PD 98059 and U0126 (2 h and 15 min of incubation, respectively), which inhibit MEK (MAPKK); and 2) a dominant-negative MEK variant that inhibits cellular MEK (see “Materials and Methods”). The results,
shown in Fig. 3, A–C, demonstrate that induction of Ha-Ras by H$_2$O$_2$ was abolished by MEK inhibition. To confirm that H$_2$O$_2$ was downstream PDGF, we treated the cells with genistein, a tyrosine kinase inhibitor, in the presence of PDGF or H$_2$O$_2$. The data shown in Fig. 3D indicate that H$_2$O$_2$ was a powerful inducer of Ha-Ras, also in the presence of genistein. As expected, the drug inhibited the induction of Ha-Ras by PDGF. However, longer incubation periods (90 min) in the presence of genistein inhibited Ha-Ras and ERK1/2 induced by H$_2$O$_2$, indicating that long term effects of H$_2$O$_2$ required active PDGF receptor (data not shown). Taken together the data, illustrated in Figs. 2 and 3, indicate that PDGF via ERK1/2 and via ROS induced Ha-Ras protein. ROS are downstream the PDGF receptor, because they induced Ha-Ras independently on the activation of the receptor.

Down-regulation of ERK1/2 (Figs. 2, D and E and 3) reduced Ha-Ras levels. Maintaining ERK1/2 high, by expressing constitutively active Ha-Ras or a dominant-negative MEK protein, ROS production was high and Ha-Ras did not decay (Fig. S2B, supplemental material). Only Ha-Ras was stabilized, since Ki-Ras levels were marginally affected by ERK1/2 inhibition in primary fibroblasts. More importantly, Ha-Ras stabilization was peculiar to primary cells, because immortalized cells such as 3T3 fibroblasts, CHO, PC12, and COS7 contained stable and high levels of Ha-Ras, which were insensitive to ERK1/2 inhibition. Also, expression of constitutive MEK-ERK2 or prolonged treatment of fibroblasts with H$_2$O$_2$ did not change Ha-Ras mRNA levels (Fig. S3, supplementary material).

The data shown above do not clearly indicate the mechanism responsible for Ha-Ras stabilization induced by PDGF-ROS-ERK1/2. To this end, we treated the cells with MG132, a widely used proteasome inhibitor and monensin, a toxin known to inhibit receptor recycling through the inhibition of endosome acidification. Fig. 3E shows that MG132 induced Ha-Ras, and its effect was not additive when administered with...
PDGF or H₂O₂. Conversely, monensin had no effect alone or with PDGF or H₂O₂ on Ha-Ras levels. Under the same conditions, Ki-Ras or PDGFR protein levels were not influenced by MG132 treatment (Fig. 3F).

Amplification of ROS-Ras Signaling in Vivo in Scleroderma Fibroblasts—To determine if the signaling pathway connecting ROS to Ras was relevant in vivo, we took advantage of fibroblasts derived from patients affected by systemic sclerosis. These cells produce high levels of ROS (12) and are subjected to constitutive stimulation in vivo of PDGF receptor antibodies.5 Fig. 4A shows that three fibroblast lines derived from these patients contain high ROS, superoxide, and H₂O₂ levels. ROS accumulation was severely inhibited by farnesyl transferase and MEK inhibitors (Fig. 4A). Moreover, these cells contained higher levels of Ha-Ras protein, compared with normal controls (Fig. 4B). Ras activity was also increased relative to normal control cells (Fig. 4C). We have recently completed the analysis of 46 patients affected by systemic sclerosis, and in all cases the ratio Ha/Ki protein levels was higher than 2.5 The analysis of downstream Ras effectors (AKT and ERK1/2) or other stress kinases (JNK) indicated that only ERK1/2 were selectively activated (Fig. 4D). ROS, Ras, and ERK1/2 activation were linked, because MEK inhibitors, ROS scavengers, or farnesyl transferase inhibitors were able to reduce Ras P-ERK1/2 and ROS levels (Figs. 3, 4A and 4B and 4A). ROS, Ha-Ras and active ERK1/2 slowly decayed in cells cultured in low serum and in 1–2 days returned to the baseline (data not shown).

Biological Consequences of Ha-Ras Stabilization in Vivo Are High ROS, High ERK, DNA Damage, Collagen Synthesis, and Senescence—We next asked if ROS-Ras amplification was affecting the phenotype of these cell lines. To this end, we determined: 1) activation of DNA damage checkpoints and chromosomal alterations; 2) stress-induced apo-
We have evidence that Ras induction by growth factors and ROS is solely regulated by GTP-GDP binding activity. Because widespread expression of Ha- or Ki-Ras is not tolerated during development or in adult organisms, in primary cells the levels of the proteins are maintained low (20).

We have replicated in normal fibroblasts all the features indicated in Fig. 5 (collagen induction, DNA damage, H$_2$O$_2$-induced apoptosis) by expressing Ha-Ras in a ratio 5:1 relative to Ki-Ras (Fig. S2, supplementary material and Ref. 15).

These data establish a link between ROS-Ras amplification and the complex phenotype of scleroderma fibroblasts in vivo. Moreover, they provide the tools for a possible diagnosis and a targeted therapy of this so far not curable illness.

DISCUSSION

The data reported here indicate a novel level of regulation of Ras proteins, so far unknown. In established and immortal cell lines, Ras is solely regulated by GTP-GDP binding activity. Because widespread expression of Ha- or Ki-Ras is not tolerated during development or in adult organisms, in primary cells the levels of the proteins are maintained low (20).

We have evidence that Ras induction by growth factors and ROS is not unique to primary fibroblasts. In peripheral lymphocytes, primary...
In primary fibroblasts, the accumulation of Ras protein is triggered by PDGF and ERK1/2 (Figs. 1 and 2). ROS induced by PDGF maintain ERK1/2 active (Fig. 2). ROS induction of Ha-Ras is independent on PDGF stimulation and can be maintained by ROS (Fig. 3). However, in the absence of PDGF stimulation, H2O2 is not able to maintain high Ha-Ras levels for longer periods (2 h).

As to the mechanism underlying Ha-Ras induction by PDGF and ROS, the data shown in Fig. 3F indicate that the Ha-Ras protein is degraded by the 26 S proteasome and that ERK1/2 protect Ha-Ras from degradation (Fig. 3, A–C). Because MG132 and PDGF effects were not additive, we suggest that PDGF via ERK1/2 inhibits proteasome degradation of Ha-Ras. At present, we do not know if proteasome directly degrades Ras protein. There is a similar example of proteasomal degradation (Fig. 3, A–C). There is a similar example of proteasomal degradation of Ha-Ras. At present, we do not know if proteasome directly degrades Ras protein. There is a similar example of proteasomal degradation of Ha-Ras.

In the presence of physiological stimuli, we believe that the regulation of Ras protein levels protects primary cells from excessive or inappropriate signals. Coupling ROS to Ras highlights the primary role of Ras proteins as sensors and regulators of redox signals. The different turn-over in primary cells and the different effects on ROS levels by Ha- and Ki-Ras (7) (Fig. S2, supplementary materials) suggest that the activation of these 2 isoforms signals to different cell compartments the type and the levels of ROS generated; i.e. the metabolic state, and availability of nutrients. Constitutive or mutational activation of Ras-ERK1/2 signaling results in loss of this type of metabolic regulation. This may explain the opposite life-span phenotypes of S. cerevisiae expressing ras2Val19 or ras2 wild-type gene (11). In primary cells, senescence or growth or differentiation are critically dependent on the integrity of this circuitry.

Acknowledgment—We thank Dr. Marcello Melone at the Institute of Physiology of the University of Ancona for help with fluorescence microscopy.

REFERENCES


FIGURE 6. Circuitry linking Ras and ROS. Schematic diagram illustrating the signaling pathway, initiated by PDGF and triggering ROS production by NADPH oxidase. ROS activates ERK1/2, which induces Ha-Ras. Once triggered, the circuitry is relatively independent on PDGF signaling. This loop is amplified in scleroderma cells.

mouse neurons and astrocytes ROS induce Ha- or Ki-Ras protein levels.6

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Amplification of ROS-Ras Signaling in Vivo—The pathway we have described is relevant in vivo, because we find that cells isolated from lesions of patients affected by systemic scleoderma, contain the elements indicated in Fig. 6. Systemic sclerosis is an autoimmune disease, characterized by extensive fibrosis of the skin and internal organs, because of exaggerated production of collagen by fibroblasts (23). Fibroblasts, derived from systemic sclerosis patients, contain high Ha-Ras and ROS levels and constitutive activation of ERK1/2. These features are the hallmarks of the signaling pathway we have described above in normal fibroblasts stimulated with H2O2. In Fig. S2 (supplementary materials) we show that we can convert normal in scleroderma fibroblasts by overexpressing Ha-Ras. Conversely, overexpression of Ki-Ras or cat- lase inhibited the scleroderma phenotype in fibroblasts, isolated from scleroderma lesions. We have recently found that the triggers of the

scleroderma phenotype are stimulating antibodies against PDGF receptor. Systemic sclerosis patients synthesize stimulating antibodies to the PDGF receptor. These antibodies stimulate fibroblasts and monocytes to produce high ROS (14), which set off ERK1/2 and induce Ha-Ras.5 Inhibition of any of the components of this loop (ERK1/2, Ras, ROS) down-regulated the system and abolished the biological effects of RAS-ROS activation, such as accelerated senescence of fibroblasts, which characterizes the phenotype of systemic sclerosis cells (23, 24). This phenotype is characterized by: 1) high susceptibility to apoptosis; 2) severe DNA damage, 3) vigorous transcription of collagen genes. In this framework, fibrosis is the consequence of loss of cells (apoptosis) and deposition of collagen (23, 24). The loop triggered initially by PDGF receptor activation, is reinforced by high ROS produced by activation of NADPH oxidase by Ha-Ras-ERK1/2 (24). Thus, inhibition of either ROS, or ERK1/2 or Ras converts scleroderma to normal fibroblasts. However, we find that PDGF signaling is required for long term ROS production, because inhibition of PDGF receptor for 4–12 h reduced Ras-ROS-ERK1/2-collagen levels (Fig. 6 and data not shown).

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6 E. Janda and A. Porcellini, personal communication.
Regulation of Ras Protein Levels in Primary Cells

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Silvia Svegliati, Raffaella Cancello, Paola Sambo, Michele Luchetti, Paolo Paroncini, Guido Orlandini, Giancarlo Discepoli, Roberto Paterno, Mariarosaria Santillo, Concetta Cuozzo, Silvana Cassano, Enrico V. Avvedimento and Armando Gabrielli

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