RESEARCH COMMUNICATION

Rac1, and not Rac2, is involved in the regulation of the intracellular hydrogen peroxide level in HepG2 cells

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In order to elucidate the components of the oxygen sensory complex in HepG2 cells which regulates the production of erythropoietin, we have microinjected recombinant variants of the human small GTP-binding protein hRac1 and measured their effects on the production of reactive oxygen species (ROS) by the dihydrorhodamine-123 technique. The dominant-negative mutant hRac1(T17N) inhibits the NADH-stimulated production of ROS in HepG2 cells, whereas the constitutively activated hRac1(G12V) leads to an increase in intracellular ROS concentration. Reverse transcriptase PCR analysis showed that the *hRac*1, but not the *hRac*2, gene is expressed in HepG2 cells. These results demonstrate that hRac1, and not hRac2, is involved in the regulation of ROS production in HepG2 cells and suggest that hRac1 specifically functions in the non-phagocytic NAD(P)H oxidase complex.

INTRODUCTION

The maintenance of oxygenation is an essential process in mammals and is regulated by several mechanisms. The glycoprotein hormone erythropoietin (Epo), produced mainly in liver and kidney, but recently also observed in brain cells [1], is one of regulators of oxygenation by inducing the production of red blood cells (see [2] for review). The production of Epo is regulated by the extracellular oxygen pressure and can be induced up to 1000-fold by hypoxia (see [3] for review). Consequently, the presence of an oxygen-sensing mechanism has been proposed (see [4] for review). Extracellular oxygen is directly related to the production of reactive oxygen species (ROS) triggering Epo production in HepG2 cells [5]. In HeLa and Hep3B cells, it was shown that the alpha-subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1) is constitutively degraded by hydroxyl radicals (•OH), whereas stimulation of the cells by hypoxia leads to lower intracellular ROS concentrations, allowing the formation of an active heterodimeric complex of the alpha- and the beta-subunit responsible for the upregulation of the Epo gene [6]. A similar hypoxia- and HIF-1-dependent expression was observed in endothelial cells for another hormone, vascular endothelial growth factor, indicating a more general mechanism [7,8].

After it became evident that a haemoprotein is involved in the oxygen-sensing signal-transduction pathway [9], the involvement of the non-respiratory cytochrome b_{558} in HepG2 cells was first proposed by Görlach et al. [10]. The cytochrome b_{558} that was found to be present in various oxygen-sensing cell types [11,12] is very similar to the cytochrome b_{558} found in the NADPH oxidase complex of neutrophils, which consists of $p22^{phox}$ and the haem-containing $gp91^{phox}$. The NADPH oxidase complex of stimulated neutrophils comprises furthermore $p67^{phox}$, $p47^{phox}$ and the small GTP-binding proteins Rac and Rap1A [13,14]. The function of an additional component, p40 [15], is still unclear. A number of these components, i.e. $gp91^{phox}$, $p22^{phox}$, $p67^{phox}$ and $p47^{phox}$, can also be detected by immunohistochemistry and molecular biology in oxygen-sensing cells, e.g. HepG2 cells, neuroepithelial bodies of the lung, smooth muscle cells of the pulmonary vasculature and type I cells of the carotid body [16–21].

Several studies have suggested that the non-phagocytic $gp91^{phox}$ is an isoform of the phagocytic protein [22–24] and that this isoform is expressed in most cell types [25]. Recently, the sequence of $gp91^{phox}$ isolated from chondrocytes was shown to differ in three amino acids from that of the phagocytic NADPH complex [26]. Interestingly, two of these differences are located in the regions of the $gp91^{phox}$ protein that supposedly interact with NADPH and $p47^{phox}$. In another recent publication, four types of polymorphism of the $p22^{phox}$ gene were noted [27]. One of these mutates His-72 into Tyr, which is located in the potential haem-binding region. These differences may explain the observation that Epo production in B-cells of chronic granulomatous patients, who suffer from mutations in the phagocytic cytochrome b_{558}, can be induced by hypoxia [28]. However, different regulatory mechanisms or expression levels or even isoforms of the other components may also cause the deviating activities of the oxidase complexes of phagocytic ('high-output') and non-phagocytic ('low-output') origin.

We were interested in the role of human small GTP-binding protein Rac (hRac) in the oxygen-sensor complex. Therefore, we have microinjected mutated variants of this protein in HepG2 cells and measured the effect on ROS production. Moreover, we determined the expression of two Rac isoforms, hRac1 and hRac2, in HepG2 cells by reverse transcriptase (RT)-PCR. We present evidence that hRac1, but not hRac2, is produced in HepG2 cells and participates in ROS production, demonstrating its involvement in the NAD(P)H oxidase complex and thus in the oxygen sensing mechanism.

MATERIALS AND METHODS

Production and purification of recombinant proteins

hRac proteins were produced as glutathione S-transferase (GST) fusion proteins from the pGEX plasmids in protease-poor

Abbreviations used: DTE, dithioerythritol; Epo, erythropoietin; GST, glutathione S-transferase; HIF-1, hypoxia-inducible factor-1; hRac, human small GTP-binding protein Rac; ROS, reactive oxygen species; RT-PCR, reverse transcriptase PCR; wt, wild type.

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Escherichia coli strain AD202 [29] according to the standard protocol for GST fusion proteins. Basically, cells were grown in Standard I medium (Merck, Darmstadt, Germany) up to an absorbance of 0.5 at 600 nm, whereafter expression was achieved by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubation at 30 °C for 5 h. Cells were harvested by centrifugation, resuspended in 3 vol. of ice-cold PBS containing 10 % (v/v) glycerol/5 mM $MgCl_2$/0.1 mM GDP/10 mM dithioerythritol (DTE) (buffer A), and lysed by sonication. The cell debris was removed by centrifugation for 1 h at 100000 ***g*** and 4 °C. A 20 ml GSH–Sepharose column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, U.S.A.) was loaded with the supernatant, washed with 100 ml of buffer A and subsequently with 50 ml of 50 mM Tris/HCl, pH 7.6/100 mM NaCl/5 mM $MgCl_2$/0.1 mM GDP/10 mM DTE/10 % (v/v) glycerol (buffer B). Finally, 300 units of thrombin (Serva, Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany) in buffer B containing 2 mM $CaCl_2$ was loaded on the column and left overnight at 4 °C. Thereafter, the cleaved protein was eluted from the column with buffer B, and, after concentration in Vivaspin vials (Vivascience Inc.), further purified on a Superdex 75 column (Pharmacia). Finally, after concentration to at least 5 mg/ml, the protein was dialysed against 50 mM Tris/HCl, pH 7.5/5 mM $MgCl_2$/5 mM DTE/0.02 mM GDP, snap-frozen in aliquots and stored at −80 °C.

During this work, we discovered by sequencing that the original pGEX plasmid carrying the supposed hRac1 wild-type (wt) gene contained a T 233 → C 233 mutation (numbering from the start codon), changing Phe-78 into Ser in the hRac1 protein (see also commentary in [30]). A new plasmid, pGEX-2T-hRac1 wt, was obtained from M. R. Webb (National Institute for Medical Research, Mill Hill, London, U.K.). Plasmids pGEX-2T-hRac1(G12V) and pGEX-2T-hRac1(T17N) were provided by A. Hall (University College London, London, U.K.), and pGEX-hRac2 by Y. Takai (Osaka University, Osaka, Japan).

Growth of HepG2 cells, micro-injection and measurement of H_2O_2 concentration by confocal microscopy

HepG2 cells (ATCC HB 8065) were cultivated in monolayer tissue culture as described by Görlach et al. [10] in RPMI 1640 medium [Life Technologies (Gibco), Eggenstein-Leopoldshafen, Germany] supplemented with 10 % (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml; Gibco) at 37 °C in an incubator containing humid air with 5 % CO_2 (Stericult 200, Labotect, Göttingen, Germany).

The micromanipulator 5171 (Eppendorf, Hamburg, Germany) together with the transjector 5246 (Eppendorf) was used for microinjection of Rac proteins into HepG2 cells grown on CellLocate coverslips (Eppendorf). The cells were impaled with commercially available glass microelectrodes having a tip diameter of 0.5 ± 0.2 μm (Femtotips, Eppendorf). An injection pressure of 6000–7500 Pa and an injection duration of 0.3 s was used. Under these conditions the microinjection did not affect the morphological appearance of the cells. To monitor the success of microinjection, the intracellular buffer (140 mM KCl/1 mM $MgCl_2$/8 mM NaCl/10 mM Hepes, pH adjusted to 7.2 with KOH) that was used for dilution of the different hRac proteins contained 50 μM SNARF calcein (Molecular Probes, Eugene, OR, U.S.A.). After microinjection, cells were incubated in RPMI 1640 medium at 37 °C for 3 h. Intracellular ROS levels were measured after an additional incubation of the cells for 45 min in medium containing 25 μM dihydrorhodamine-123 (Molecular Probes)[10]. Dihydrorhodamine-123 is described to react specifically with H_2O_2. It is well established in the literature, however, that the reaction with H_2O_2 is rather slow under ambient conditions. It has been shown that the addition of iron compounds yields a fast oxidation of dihydrorhodamine-123 to fluorescent rhodamine-123 [31]. This suggests the involvement of the Fenton reaction in the activation process of dihydrorhodamine-123. A Fenton-reaction oxidation involves several steps with well-characterized kinetics. One important intermediate is the hydroxyl radical which has been detected by several spectroscopic methods [32]. Ehleben et al. [20] proposed the following chemical reaction for the conversion of non-fluorescent dihydrorhodamine-123 into rhodamine-123: the reaction of the $^{\bullet}OH$ with dihydrorhodamine-123 yields a tertiary free radical which is known to be rather stable. The radical is able to give a rearrangement of the π system, yielding fluorescent rhodamine-123. The electron in excess can either be abstracted by another $^{\bullet}OH$, yielding an hydroxyl anion (OH^-), or by H_2O_2, yielding $^{\bullet}OH$. The latter reaction pathway would be the starting point for a new cascade.

For visualization of the rhodamine-123 fluorescence inside HepG2 cells as an indication of the intracellular H_2O_2 level, a commercial confocal laser scanning microscope was used (MRC 600; Bio-Rad Inc., Hempel Hempstead, U.K.) attached to an inverted microscope (ICM 405; Zeiss, Cologne, Germany) having a Plan-Achromat 32× objective (Zeiss). The two dyes Rhodamine-123 and SNARF calcein were excited with the 488 nm line of an Ar^{2+} ion laser (Omnichrome; Laser 2000, Wessling, Germany) and the fluorescence was detected on two separate channels using band-pass filters of 570 nm and 640 nm respectively. The fluorescence intensity of the individual microinjected HepG2 cells was measured with the MRC600 software.

Data are given as means ± SD. For statistical analysis Student's *t*-test was performed. Differences were considered significant when the P value was < 0.05.

RT-PCR and PCR sequencing

HepG2 cells (approx. 1.5×10^7 cells) were washed with PBS, suspended by incubation with trypsin/EDTA, pelleted by centrifugation and washed with PBS. RNA was prepared from these cells with the RNeasy kit (Qiagen, Hilden, Germany), according to the supplier's instructions. The concentration of the RNA solution was determined spectrometrically (absorbance at 260 nm). The isolation of RNA from purified human lymphocytes (donated by Dr. N. Petersen, Institut für Blutspendewesen, Dortmund, Germany) was carried out in an identical manner.

The production of cDNA was carried out using an RT-kit (Promega, Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany) by incubating 1 μg of RNA in 20 μl of reverse transcriptase buffer containing 1 mM of each dNTP, 2 units of Rnasin, 25 μg of oligo(dT), 5 mM $MgCl_2$ and 13.5 units of avian myeloblastosis virus reverse transcriptase for 35 min at 42 °C, after which the enzymic activity was terminated by heat treatment (3 min at 95 °C).

Half of the RNA/cDNA mixture (10 μl) was incubated in 50 μl of PCR buffer (Qiagen) containing 0.4 μM hRac 5′-primer (5′-GGAATTCTATGCAGGCCATCAAGTGTGT-3′; the underlined sequence is identical with the 5′-end of both *hRac1* and *hRac2*) and 0.4 μM hRac1 3′-primer (5′-CAGAAGCTTTTA-TTACAACAGCAGGCATTTT-3′) or of the hRac2 3′-primer (5′-ACAGGTCGACTTACTAGAGGAGGCTGCAGGCG-3′) (the underlined sequences are complementary to the 3′-ends of the *hRac1* or *hRac2* coding sequences respectively) and 2.5 units of Taq polymerase (Qiagen) for 30 cycles using the following profile: 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. As a control, the same incubation was performed with 0.5 μg of RNA

that was not used for the reverse transcriptase isolation of cDNA, and with 0.2 mM of each dNTP. A 10 μl volume of each mixture was loaded on an agarose gel.

RESULTS AND DISCUSSION

In the present study, we have measured the influence of microinjected hRac1 proteins on the concentration of H_2O_2 in HepG2 cells by the Rhodamine-123 method. The conversion of dihydrorhodamine-123 by H_2O_2 into the fluorescent Rhodamine-123 by the Fenton reaction [31] occurs close to the nucleus and might be linked to Fe–S clusters [20]. In each experiment the fluorescence of the individual cells was measured relative to the cell which was microinjected with hRac1(F78S) and which showed the highest fluorescence within the focus of the laser beam. To show clearly the response of the low-output NAD(P)H oxidase, rotenone (50 μM) was used to suppress ROS formation by the respiratory chain and NADH (1 mM) was applied extracellularly to the cells to stimulate the cell-membrane-located NAD(P)H oxidase throughout the experiments [20]. The fluorescence of HepG2 cells microinjected with 200 μM wt hRac1(F78S) was significantly stronger than that of HepG2 cells microinjected with 200 μM dominant-negative hRac1(T17N) protein (Figure 1). Cells microinjected with hRac1(F78S) and hRac1 wt showed an identical fluorescence yield (results not shown), indicating that the mutation does not affect the action of hRac1 on NAD(P)H oxidase activity, as already proposed by Hirsberg et al. [30]. No significant difference was observed between the fluorescence signal of non-injected cells and cells injected with buffer, nor between the fluorescence signal of non-injected cells and cells injected with wt hRas(F78S) (results not shown).

The oncogenic hRac1(G12V) stimulated H_2O_2 production by nearly 20 % more than wt hRac1(F78S). The stimulatory action of hRac1(G12V) was abrogated by addition of diphenylene iodonium chloride. This substance is a well-known inhibitor of flavoproteins involved in NADPH oxidase activity and of hypoxia- or cobalt-induced Epo production [33,34]. Microinjection of the dominant-negative mutant hRac1(T17N) caused an inhibition of the H_2O_2 production in HepG2 cells to 60 % of the reference value (Figure 1). This inhibition was also observed after microinjection of a lower concentration (100 μM) of hRac1(T17N) (results not shown). The stimulatory and inhibitory actions of the activated and the dominant-negative hRac1 mutants on the H_2O_2 level are similar to their effects on NADPH oxidase activity in fibroblasts [35]. Our results thus show that hRac1 plays an essential role in ROS production in HepG2 cells and supports the hypothesis that an NAD(P)H oxidase complex is involved in the oxygen-sensitive low-output-ROS production in HepG2 cells.

Lately, there has been a growing interest in the non-phagocytic NAD(P)H oxidase complex and the active role of ROS in a variety of cellular responses (see [36,37] for reviews). As pointed out above, isoenzymes from the proteins involved in the phagocytic NADPH oxidase complex might be involved in the non-phagocytic complex. In this light, hRac2 was found to be a stronger stimulator than hRac1 of the phagocytic NADPH oxidase [38] and to interact more strongly than hRac1 with the phagocytic $p67^{phox}$ in double hybrid analysis [39]. Didsbury et al. [40] detected a weak signal for hRac1 and no signal for hRac2 in liver cells using Northern analysis. In order to expand these observations, we have checked the expression of the *hRac1* and *hRac2* genes in HepG2 cells and lymphocytes by the more sensitive RT-PCR technique. As shown in Figure 2, in HepG2 cells only hRac1 mRNA can be demonstrated, whereas lymphocytes express both hRac1 and hRac2. The PCR products correspond to the length of the hRac genes (579 bp). PCR sequencing of the hRac1 RT-PCR product in HepG2 cells showed that the product is indeed hRac1 (results not shown). Together with the fact that, by Northern blotting, hRac2 mRNA was found to be particulary abundant in cells of myeloid origin,

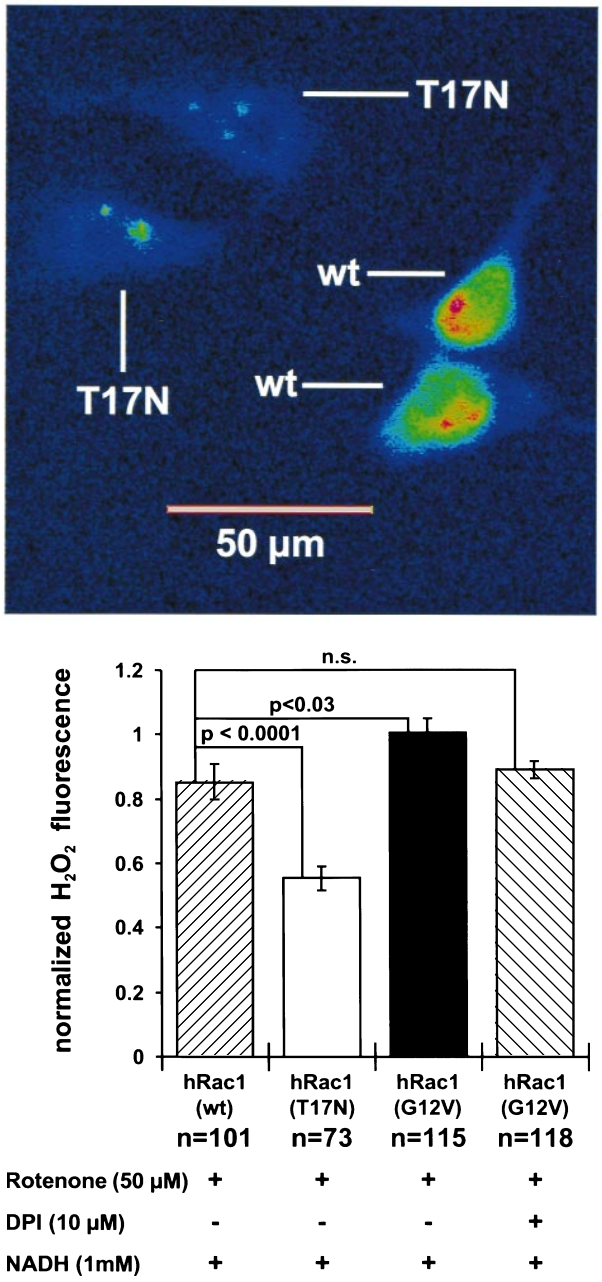

Figure 1 Confocal analysis of the effect of microinjection of Rac proteins on H_2O_2 production in HepG2 cells

Top: Rhodamine-123 fluorescence intensity of cells microinjected with 200 μM of wt hRac1(F78S) (indicated wt) or the dominant-negative mutant hRac1(T17N) (indicated T17N). Low Rhodamine-123 fluorescence (i.e. low H_2O_2 generation) is shown in blue, whereas medium and high fluorescence yields are depicted in green and red respectively. Bottom: statistical analysis of Rhodamine-123 fluorescence in HepG2 cells microinjected with 200 μM of different hRac1 proteins. The oncogenic protein hRac1(G12V) is indicated as G12V. In all experiments, 50 μM rotenone and 1 mM NADH were added to the medium. n = number of cells; p = significance level; n.s. = not significant; DPI, diphenylene iodonium chloride.

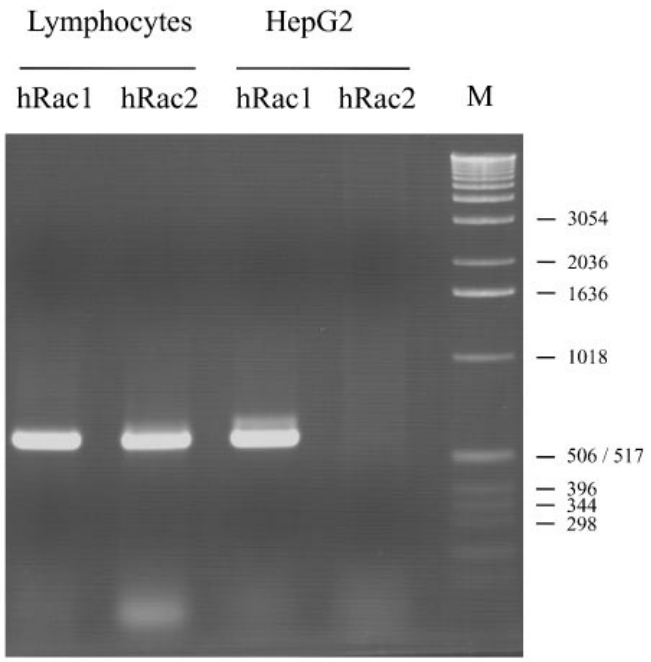

Figure 2 RT-PCR analysis of the expression of Rac genes in human lymphocytes and HepG2 cells

Bands at approx. 580 bp indicate the presence of hRac mRNA in the cells. M, marker DNA (1 kb DNA Ladder; Gibco-BRL Life Technologies).

whereas hRac1 was expressed ubiquitously [40], these results strongly suggest that hRac1 regulates the low-output NAD(P)H oxidase complex, whereas hRac2 is essential for the phagocytic high-output NADPH oxidase activity. It remains to be seen whether the recently discovered third Rac isoform, Rac3 [41], can also play a role in the NADPH oxidase complex.

In conclusion, our results strengthen the model that a low-output NAD(P)H oxidase complex is involved in the generation of ROS in HepG2 cells and demonstrate that hRac1, rather than hRac2, plays an essential role in the regulation of its activity.

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