Enhancement of Protein Kinase C-dependent O2 Production in Epstein-Barr Virus-transformed B Lymphocytes by p120Ras-GAP Antisense Oligonucleotide*

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The mammalian Ras GTPase-activating protein (p120Ras-GAP) interacts with activated members of the Ras superfamily of GTP-binding proteins to accelerate their deactivation by sharply increasing their rates of GTP hydrolysis. Among the Ras-family proteins interacting with p120Ras-GAP is Rap1A/Krev1, whose activity is not affected by p120Ras-GAP but which competes with Ras for p120Ras-GAP. A second protein that interacts with p120Ras-GAP is p190Rac-GAP, which activates the GTPase of guanine nucleotide-binding proteins of the Rho family (including Rac1 and Rac2). Both these p120Ras-GAP-binding proteins are of interest in connection with the regulation of the respiratory burst oxidase, Rap1A/Krev1 because it copurifies with cytochrome b558, and p190Rac-GAP because it inhibits the Rac2-dependent activation of the respiratory burst oxidase in a cell-free system. Using an 18-mer antisense oligonucleotide, we were able to decrease the expression of p120Ras-GAP in Epstein-Barr virus-transformed B lymphocytes. Under conditions where p120Ras-GAP expression was significantly depressed by antisense oligonucleotides, we observed a 40% increase in protein kinase C-dependent but not receptor-dependent O2 production. In contrast, sense and scrambled oligonucleotides had no effect on either p120Ras-GAP expression or O2 production. Our results suggest a role for p120Ras-GAP as a negative regulator in the protein kinase C-mediated activation of the respiratory burst oxidase.

The products of the mammalian N-, K-, and Ha-ras genes are 21-kDa guanine nucleotide-binding proteins that possess a low intrinsic GTPase activity (1, 2). They play a significant role in both the control of normal cell growth (3) and its malignant subversion (4) and have been implicated as key elements in signal transduction in lymphoid and myeloid cell lines (5–7). The activity of Ras depends on the ratio of bound GTP to GDP, a value that is determined by certain regulatory proteins: guanine nucleotide exchange factors such as Sos (8) and Vav (9), which activate Ras by catalyzing the exchange of bound GDP for GTP, and GTPase-activating proteins such as the NF-1 gene product (10) and p120Ras-GAP, which deactivate Ras by stimulating the conversion of bound GDP to GDP (11).

p120Ras-GAP is a monomeric 120-kDa cytosolic protein that greatly accelerates the conversion of p21Ras-GTP to the conformationally inactive GDP-bound form (12, 13). Accordingly, p120Ras-GAP has been proposed as a negative regulator of Ras in cellular processes. This proposal is consistent with the finding that overexpression of p120Ras-GAP blocks oncogenic transformation (14, 15) and inhibits Ras-dependent cellular signaling (16). In addition, since the site of interaction of p21Ras-GTP with p120Ras-GAP overlaps the effector region of Ras, it has been proposed that p120Ras-GAP may also serve as an effector protein of active Ras (17, 18).

p120Ras-GAP consists of a C-terminal domain that interacts with Ras and an N-terminal portion containing two Src homology (SH2) domains and one intervening SH3 domain (reviewed in Ref. 19). The p21Ras-GTP-p120Ras-GAP complex may interact with downstream effectors via these Src-homology domains. SH2 domains bind phosphotyrosine residues and have been implicated in the interactions of tyrosine kinases of the Src family with their effector proteins (20, 21). Several members of the Src family of protein-tyrosine kinases (e.g. c-Src and Lck) have been shown to associate with and phosphorylate p120Ras-GAP (22–24). In fibroblasts transformed by cytoplasmic and receptor-like tyrosine kinases (25), p120Ras-GAP forms complexes with two phosphotyrosine-containing proteins, p62 and p190Rac-GAP. The complex with p62 appears to involve the SH2 domains of p120Ras-GAP (26, 27), and the formation of the p120Ras-GAP/p190Rac-GAP complex is dependent on phosphorylation (28), suggesting that it too involves the p120Ras-GAP SH2 domains. The complex itself has diminished p120Ras-GAP activity in vitro (28). Tyrosine-phosphorylated p62 and p190Rac-GAP have been detected in anti-p120Ras-GAP immunoprecipitates from various activated or transformed B- and T-cell lines (30, 31), and were proposed to participate in signal transduction in these cells. The p120Ras-GAP-associated p190Rac-GAP stimulates the intrinsic GTPase activity of Rac1 and Rac2, which are Rho family guanine nucleotide-binding proteins (32). In human phagocytes, Rac2 plays a significant role in the activation of the respiratory burst oxidase (33–35). The human oxidase consists of two membrane-bound components (gp91phox and p22phox) and at least three cytosolic components (p47phox, p67phox, and Rac2) that after cellular activation assemble to form the active enzyme (reviewed in Ref. 36). p190Rac-GAP has been shown to inhibit the Rac2-dependent activation of the respiratory burst oxidase in a cell-free system (37), pointing to a role for this p120Ras-GAP-associated protein in the regulation of the oxidase. The same oxidase has also been found in normal human B-cells and EBV-transformed B lymphocytes (38, 39), and Rac2 plays

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The abbreviations used are: EBV, Epstein-Barr virus; phorbol, phorbol myristate acetate; HBSS, Hank’s balanced salts solution without calcium or magnesium; GFX, GF109203X; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
a significant role in the activation of the lymphocyte oxidase (33). Since we were able to detect p190Rac-GAP in p120Ras-GAP immunoprecipitates from cultured EBV-transformed B lymphocytes and since there have been several reports of the use of antisense oligonucleotides in these cells (33, 40, 41), we used antisense oligonucleotides to study the effects of p120Ras-GAP (and possibly associated proteins) on O2 production. In this paper we describe the results of this study.

MATERIALS AND METHODS

Reagents—RPMI 1640 medium was purchased from Bio-Whittaker (Walkersville, MD). Hank’s balanced salt solution (HBSS), cytochrome- methione-free DMEM, DMEM, and Opti-MEM medium were from Life Technologies, Inc. Fetal calf serum was purchased from Hyclone (Walkersville, MD). Hanks’ balanced salt solution (HBSS), cysteine/hydrochloride, and fetal calf serum were purchased from UBI (Lake Placid, NY). Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma. The monoclonal anti-p120Ras-GAP antibody was obtained from Sigma.

Enhancement of O2 Production by p120Ras-GAP Antisense

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with $^{32}$P, we showed that the added oligonucleotides were taken up by the cells in a time-dependent fashion, with maximum uptake at 4 h as previously reported (40) (data not shown). In addition, we found by HPLC that the oligonucleotide was not significantly degraded after culture for 24 h in medium alone and that even when cultured in the presence of the cells, 20% of the oligonucleotide initially added was present after 24 h (Fig. 2).

When the cells were incubated for 24 h in the presence of the

antisense oligonucleotide, expression of $p120^{\text{ras-GAP}}$ was inhibited (Fig. 3). Immunoblots of cells treated with the antisense oligonucleotide showed a substantial reduction in the level of $p120^{\text{ras-GAP}}$ as compared with untreated cells or cells treated with sense or scrambled oligonucleotides. By contrast, the levels of $p47^{\text{phox}}$ and $p67^{\text{phox}}$ were not significantly altered by incubation with antisense oligonucleotide, as revealed by parallel immunostaining of blots with specific antibodies against these two proteins. These results indicated that the suppression of $p120^{\text{ras-GAP}}$ expression by the antisense oligonucleotide was specific and did not affect the expression of two irrelevant proteins related to the respiratory burst oxidase in this cell type.

$O_2^{-}$ production by EBV-transformed B-cells treated with antisense, sense, or scrambled oligonucleotides was detected by luminol-enhanced chemiluminescence of cells stimulated with phorbol, Pansorbin, or ionomycin. When antisense-treated cells were stimulated with phorbol we observed a significant enhancement of $O_2^{-}$ release in comparison with cells preincubated with sense or scrambled sequences (Fig. 4, top). This enhancement always correlated with a reduced expression of $p120^{\text{ras-GAP}}$ and was not observed in cells incubated in the presence of the oligonucleotide for 3 days, conditions under which the expression of $p120^{\text{ras-GAP}}$ had returned to levels seen after incubation with sense or scrambled sequences (data not shown). At 10 min, burst enhancement averaged $147 \pm 10\%$ (S.E.) in cells treated with 0.1 mM antisense oligonucleotide. When $O_2^{-}$ formation was triggered by Pansorbin, however, which acts through the cell’s surface immunoglobulin receptors, the burst-enhancing effect of antisense oligonucleotides was not seen (Fig. 4, middle).

$O_2^{-}$ production by Pansorbin-treated cells was much lower than that of phorbol-treated cells. To determine whether the difference in the effect of antisense oligonucleotides on phorbol-stimulated versus Pansorbin-stimulated cells was due to the weakness of the latter stimulus, antisense experiments were carried out on cells treated with ionomycin, another weak stimulus. Experiments with the protein kinase C inhibitor GFX showed that ionomycin, like phorbol but unlike Pansorbin, activated $O_2^{-}$ production by EBV-transformed B lymphocytes through a protein kinase C-dependent mechanism (Fig. 5). Although levels of $O_2^{-}$ production in response to ionomycin were
Similar to those seen with Pansorbin, enhanced O$_2^*$ production in response to ionomycin was increased to 133 ± 8% (S.E.) of control by the antisense oligonucleotide.

p120$^{\text{ras-GAP}}$ has been connected to tyrosine kinase-mediated signaling pathways, where it is thought to function downstream of activated Src-like protein tyrosine kinases or growth factor receptor tyrosine kinases (49, 56). In T-cells, p120$^{\text{ras-GAP}}$ is bound to Lck and becomes specifically phosphorylated by this Src-like tyrosine kinase (23). Since Lck has been reported to play a significant role in EBV-induced growth of transformed B lymphocytes (40), and since antisense oligonucleotides against Lck inhibited the growth of EBV-transformed B-cells, we examined the effects of the p120$^{\text{ras-GAP}}$ antisense oligonucleotide on this parameter. We found that when p120$^{\text{ras-GAP}}$ expression was significantly reduced, the cell count after 24 h was low (Fig. 6). Despite this decrease in cell proliferation, O$_2^*$ production was increased in cells incubated with the antisense oligonucleotide.

**DISCUSSION**

Antisense strategy is a useful approach for elucidating the role of different proteins in signal transduction (46). Using an 18-mer antisense oligonucleotide spanning the start codon of the p120$^{\text{ras-GAP}}$ message, we examined the function of p120$^{\text{ras-GAP}}$ in the control of respiratory burst oxidase activity in EBV-transformed B lymphocytes. In agreement with Cheung and Dosch (40), we observed that these cells took up oligonucleotides in a time-dependent fashion and that the antisense oligonucleotide, but not the sense or scrambled oligonucleotide, was able to reduce the expression of p120$^{\text{ras-GAP}}$. When p120$^{\text{ras-GAP}}$ expression was reduced, O$_2^*$ production by phorbol- and ionomycin-stimulated lymphocytes increased, although O$_2^*$ production by protein A (Pansorbin)-stimulated cells remained unchanged. Both phorbol- and ionomycin-stimulated O$_2^*$ production, but not the Pansorbin-triggered burst, appear to be dependent on the activation of protein kinase C, as indicated by results obtained with GFX. The antisense results, therefore, point to a specific role for p120$^{\text{ras-GAP}}$ in the protein kinase C-dependent activation of the respiratory burst oxidase in EBV-transformed B-cells.

Several laboratories have presented evidence showing a link between p120$^{\text{ras-GAP}}$ and protein kinase C-dependent signaling pathways. In T-cells, an increase in the active p21$^{\text{ras}}$ that was induced by phorbol but not by receptor-dependent stimuli has been related to a protein kinase C-mediated inactivation of p120$^{\text{ras-GAP}}$ (7). In fibroblasts, overexpression of p120$^{\text{ras-GAP}}$ inhibited the activation of mitogen-activated protein kinase by phorbol but not by receptor-dependent stimuli (16), presumably by causing a selective blockade of signals from activated protein kinase C due to the inactivation of p21$^{\text{ras}}$. Thus, for both cell types, p120$^{\text{ras-GAP}}$ has been proposed as a negative regulator in a protein kinase C-dependent signaling pathway. Our results point to a similar role for p120$^{\text{ras-GAP}}$ as a down-regulator of a protein kinase C-dependent signaling pathway leading to an activated NADPH-oxidase.
Among the factors that regulate the activity of small guanine nucleotide-binding proteins is p190Rac-GAP, a 190-kDa protein that accelerates the deactivation of guanine nucleotide-binding proteins of the Rho class (32). p190Rac-GAP is of interest in regard to oxidase activation because Rac2, one of its targets, is known to participate in the activation of the respiratory burst oxidase both in the cell-free system (47, 48) and in whole cells (33). In accord with this idea, p190Rac-GAP was shown to inhibit Rac2-dependent oxidase activation in the cell-free system (37, 49). p190Rac-GAP is known to associate with p120Ras-GAP in many cell types (25, 26, 30, 49) and using anti-phosphotyrosine antibodies, we found a phosphorylated 190-kDa protein, which could be identified as p190Rac-GAP, in immunoprecipitates of p120Ras-GAP from EBV-transformed B lymphocytes (data not shown). The possibility therefore exists that the deficiency of p120Ras-GAP induced by the antisense oligonucleotide resulted in a delay in the deactivation of Rac2. Unfortunately, we were unable to detect p190Rac-GAP on Western blots of extracts from oligonucleotide-treated cells, so we have no direct evidence as to the effect of the antisense oligonucleotide on p190Rac-GAP concentrations in the transformed lymphocytes. However, our finding that phorbol-dependent but not receptor-dependent oxidation is affected by the antisense oligonucleotide could result from interactions between p120Ras-GAP and proteins other than p190Rac-GAP or p62, the two proteins that are commonly found in p120Ras-GAP immunoprecipitates. The Src-like tyrosine kinase Lck is a candidate of particular interest, since Lck forms a complex in vitro with the phosphorylated N-terminal SH2 domain of p120Ras-GAP (23) and is thought to play a significant role in the transformation of EBV-transformed B lymphocytes (40). The inhibition of cell growth caused by p120Ras-GAP antisense oligonucleotides might somehow be connected to the interaction between p120Ras-GAP and Lck.

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