Characterization of the Regulation and Functional Consequences of p21\textsuperscript{ras} Activation in Neutrophils by Antineutrophil Cytoplasm Antibodies

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Antineutrophil cytoplasm antibodies (ANCA) are implicated in the pathogenesis of systemic vasculitis. ANCA are directed against antigens expressed on the surface of cytokine-primed neutrophils. It was shown previously that whole IgG ANCA and its fraction antigen binding [F(ab')\textsubscript{2}] fragment can activate the GTPase p21\textsuperscript{ras}. This study shows a functional involvement of this molecule in the ANCA activation of neutrophils by inhibiting the production of superoxide with farnesylthiosalicylic acid. Using the ras activation assay, farnesylthiosalicylic acid inhibits p21\textsuperscript{ras} binding to its substrate at comparable concentrations to those seen for superoxide inhibition. It is also shown that activation of p21\textsuperscript{ras} by ANCA is transient, peaking at 5 to 10 min and returning to baseline by 30 min. The use of ras isoform–specific antibodies in Western blots established, for the first time, that Harvey-ras is not present in human neutrophils, but both Kirsten-ras (K-ras) and Neuronal-ras are. Stimulation with ANCA is able to differentially activate K-ras without effects on neuronal-ras. The activation of p21\textsuperscript{ras} by ANCA and its F(ab')\textsubscript{2} is prevented by inhibition of both Src kinases and phosphatidylinositol-3-kinase, indicating a cooperative role for both.

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p21\textsuperscript{ras} activated by ANCA during neutrophil responses. We characterized the isoforms of p21\textsuperscript{ras} present in human neutrophils and determined which have been activated by ANCA. We investigated the upstream regulators of p21\textsuperscript{ras} activation and also looked at the functional consequences of this activation in terms of induction of the respiratory burst. Despite the fact that neutrophils contain both K-ras and N-ras (but not H-ras), ANCA differentially activates only K-ras. Activation of p21\textsuperscript{ras} is controlled by both PI3K and Src-family kinases. Functionally, we demonstrated that p21\textsuperscript{ras} is involved in the ANCA-induced production of superoxide.

Materials and Methods

Protein G–sepharose columns, ECL plus, ECL, Percoll, and sheep anti-mouse horseradish peroxidase (HRP)-conjugated secondary and Rainbow markers were from Amersham-Pharmacia Biotech (Little Chalfont, UK). Hanks’ balanced salt solution and HEPES were from Invitrogen (Paisley, Scotland, UK). Anti-Ras and Ras-binding domain of human Raf-1 conjugated to agarose beads were from Upstate (Milton Keynes, UK). PP2, wortmannin, LY294002, and radicicol were from Merck Biosciences (Nottingham, UK). Anti-GST, formyl–Met–Leu–Phe (MLP), and all other chemicals were from Sigma (Poole, Dorset, UK).

Isolation of Neutrophils

Blood was obtained from healthy volunteers, and neutrophils were separated as described previously using centrifugation over a Percoll discontinuous density gradient (8). Viability was measured by the ability to exclude the dye Trypan Blue.

Preparation of ANCA and Normal IgG

Serum samples were obtained from four PR3-ANCA IgG-positive patients with Wegener’s granulomatosis, four MPO-ANCA IgG-positive patients with microscopic polyangiitis, and four healthy volunteers. The patients all fulfilled Chapel Hill definitions (9). IgG was prepared using selection on protein G–sepharose columns. Protein concentrations were estimated by spectrophotometry. All preparations were endotoxin-free as determined by Limulus amoebocyte assay.

Preparation of F(ab’\textsubscript{2})\textsubscript{2} Fragments

F(ab’\textsubscript{2})\textsubscript{2} fragments were prepared as described previously (3). F(ab’\textsubscript{2})\textsubscript{2} fragments were still able to bind ethanol-fixed neutrophils and recognized their antigen as determined by ELISA. Concentrations were used in experiments at equivalent molarity.

Superoxide Assay

Superoxide production was measured by the superoxide dismutase inhibitable reduction of ferricytochrome C as described previously (10). Neutrophils were resuspended in Hanks’ balanced salt solution that contained 10 mM HEPES (pH 7.4) at a concentration of 2 × 10\textsuperscript{6} cells/ml and primed with 2 ng/ml TNF-α and 5 μg/ml cytochalasin B for 15 min at 37°C. Aliquots of 10\textsuperscript{5} cells were then stimulated with either 1 μM fMLP or 200 μg IgG, and superoxide release was measured over 120 min. All samples were tested in triplicate, and experiments were repeated six times with at least three different neutrophil donor and IgG combinations.

Ras Activation Assay

Neutrophils were resuspended at a concentration of 10\textsuperscript{7} cells/ml in Hanks’ balanced salt solution that contained 10 mM HEPES and treated with 10 μM PP2 for 30 min, 50 μM LY294002 for 30 min, 10 nM wortmannin for 5 min, 200 ng/ml radicicol for 2.5 h, or 0 to 100 μM farnesylthiosalicylic acid (FTS) for 2.5 h followed by priming with 2 ng/ml TNF-α for 15 min at 37°C. Aliquots of 1 ml were then treated with 250 μg/ml IgG or a molar equivalent of F(ab’\textsubscript{2})\textsubscript{2} (167 μg). Cells were pelleted, snap-frozen, then resuspended in 1× lysis buffer that contained magnesium and protease inhibitors (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% vol/vol Igepal CA-630, 10 mM MgCl\textsubscript{2}, 1 mM EDTA, 10% vol/vol glycerol, 20 mM NaF, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM PMSF). Extraction proceeded for 10 min at 4°C, insoluble matter was removed, and lysates were incubated with 10 μg of Ras-binding domain of human Raf-1 conjugated to agarose beads for 30 min at 4°C. Beads were subsequently washed with lysis buffer, and active Ras was eluted by boiling in 2× sample buffer (0.125 M Tris, 20% vol/vol glycerol, 4% wt/vol SDS, 0.2 M dithiothreitol, and 0.025 mg/ml bromophenol blue).

Western Blotting

Samples were resolved on a 12% separating gel, transferred to polyvinylidene difluoride membrane, blocked with 5% milk solution/Tris-buffered saline/0.1% Tween 20 for 3 h, and exposed to anti-Ras at 1 μg/ml overnight at 4°C. After application of a secondary sheep anti-mouse HRP-conjugated antibody (1:2000, 45 min), bands were visualized by ECL plus. Blots were then stripped and reprobed with anti-GST antibody 1:20,000 overnight at 4°C. After application of a secondary sheep anti-mouse HRP-conjugated antibody, bands were visualized by ECL.

Isoform Studies

Neutrophils (5 × 10\textsuperscript{6}) were resuspended directly in boiling 2× sample buffer, sonicated on ice for 10 min, heated to 100°C for 5 min, and then further diluted in 1× sample buffer (2× sample buffer diluted 1:1 with distilled water). In addition, 4 × 10\textsuperscript{6} neutrophils/sample were stimulated with 250 μg/ml ANCA for 10 min, lysed, and treated as for the ras activation assay above, except that 40 μl of Ras-binding domain of human Raf-1 conjugated to agarose beads was used. Active Ras was eluted by boiling in 2× sample buffer, and the sample was divided equally between the lanes of an SDS-PAGE gel. Western blotting was completed as above, and blots were probed with isoform-specific antibodies. Before use, isoform-specific antibodies were titrated using dot blots so as to recognize 100 ng of standards with no crossover between isoforms (data not shown). Final concentrations used were 1:150 for anti-K-Ras, 1:10,000 for anti-H-Ras, and 1:750 for anti-N-Ras.

Densitometric Analysis

Western blots were analyzed semiquantitatively using AlphaEase software, version 3.3b (Alpha Innotech Corp., San Leandro, CA). Values are expressed as individual density values.

Statistical Analyses

Results were analyzed for statistical variance using a two-way ANOVA with repetitions.

Results

Primed neutrophils were stimulated for increasing periods of time with 250 μg of whole ANCA IgG and then subjected to the ras activation assay (Figure 1A). No activity was seen in neutrophils that were not exposed to ANCA or incubated with normal IgG at an equivalent concentration to that used for ANCA. ANCA induction of p21\textsuperscript{ras} activity was transient, commencing at 0.5 min, peaking at 5 to 10 min, and returning to
basal by 30 min. Densitometry confirmed this. The time course of superoxide production by ANCA stimulation was assessed and found, as in our previous publications, to commence after 15 to 20 min, unlike the fMLP response, which is more rapid (Figure 1B). Incubation with normal IgG gave no superoxide production.

Neutrophils were preincubated with increasing concentrations of FTS before stimulation with 250 μg of whole ANCA IgG and performance of the ras activation assay (Figure 2A). FTS had no effect on cell viability at the highest concentrations as judged by Trypan blue exclusion (97.5 ± 0.26% viable). FTS was able to abrogate the response, returning to basal activity at the higher concentrations. In the superoxide assay (Figure 2B), ANCA IgG induced a response (8.09 ± 1.1 nmol SO₂⁻/10⁵ cells) above unstimulated cells (1.59 ± 0.26 nmol SO₂⁻/10⁵ cells), which was reduced to basal levels at a concentration of FTS between 25 μM (2.55 ± 1.24 nmol SO₂⁻/10⁵ cells) and 50 μM.

Figure 1. (A) Time course of ras activation by antineutrophil cytoplasm antibodies (ANCA). Neutrophils were primed with TNF-α, then stimulated with 250 μg of ANCA from 0.5 to 30 min before the ras activation assay, blotting, and probing with anti-ras. A portion of the sample was run on a separate blot and probed with anti-ras to indicate equal loading (bottom). Time in minutes above each lane. C, untreated cells incubated for 10 min; N, 250 μg of normal IgG for 10 min. Results are representative of three independent experiments using various combinations of neutrophil donors and ANCA preparations. Densitometric analysis was performed on four independent experiments; time zero was assigned an arbitrary value of 100%, and all other values are compared with this. (B) Time course of superoxide production by ANCA. Neutrophils were incubated with 250 μg of IgG, 1 μM fMLP, or nothing, and the superoxide assay was performed as described in the Materials and Methods section (n = 3).

Figure 2. (A) Effects of farnesylthiosalicylic acid (FTS) on ras activation by ANCA. Neutrophils were isolated and preincubated with 0 to 100 μM FTS for 2.5 h at 37°C before priming with TNF-α and incubation with 250 μg of ANCA for 10 min at 37°C. Subsequently, the ras activation assay was performed and samples were subjected to Western blotting as described. Concentrations of FTS above each lane A+, ANCA plus; C0, unstimulated neutrophils; C100, unstimulated neutrophils + 100 μM FTS; f, 1 μM fMLP for 30 s. Blots were subsequently stripped and reprobed with anti-GST to indicate equal loading (bottom). Results are representative of three independent experiments using various combinations of neutrophil donors and ANCA preparations. Densitometric analysis of the individual density value of each band is displayed with unstimulated value subtracted. (B) Effect of ras inhibition on induction of superoxide production by ANCA. Neutrophils were treated with 0 to 100 μM FTS for 2.5 h at 37°C before the superoxide assay as described in the Materials and Methods section. Mean ± SEM; n = 6; *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA.
(0.61 ± 0.28 nmol SO\(^{-} \)/10\(^{5}\) cells). Normal IgG gave 1.34 ± 0.67 nmol SO\(^{-} \)/10\(^{5}\) cells (data not shown). FTS had no effect on basal superoxide release at any concentration (data not shown).

Decreasing dilutions of whole-cell lysates of human neutrophils were probed with antibodies to the three isoforms of p21\(^{ras}\) (Figure 3A). Both K-ras and N-ras were detectable at dilutions of 0.5 \(\times\) 10\(^{6}\) cells, but at no concentration was H-ras detectable. As anti-isoform antibodies had been titrated to recognize equal amounts of their standards with no crossover between isoforms, it can be determined that there was a higher amount of N-ras present in the neutrophils than K-ras. Neutrophils were stimulated with 250 \(\mu\)g of whole ANCA IgG for 10 min and then subjected to the ras activation assay. Each sample was split equally and probed for individual isoform activation.

No activation was observed for N-ras. K-ras, however, was activated by ANCA (Figure 3B).

Neutrophils were preincubated with either 50 \(\mu\)M LY294002 for 30 min or 10 nM Wortmannin (inhibitors of PI3K) for 5 min before treatment with 250 \(\mu\)g f ANCA or equivalent F(ab\(^{\prime}\))\(_{2}\), followed by the ras activation assay. Neither inhibitor had any effect on viability (LY294002 98.5 ± 1.54% or Wortmannin 98.6 ± 1.5% viable), total cellular p21\(^{ras}\) (data not shown), or basal p21\(^{ras}\) activity, but LY29400 was able to decrease the activity observed with whole ANCA (Figure 4A) and wortmannin completely abolished it (Figure 4B). In addition, F(ab\(^{\prime}\))\(_{2}\) stimulates p21\(^{ras}\), and this was also sensitive to inhibition of PI3K by LY294002 (Figure 4C).

Neutrophils were preincubated with either 10 \(\mu\)M PP2 (Src

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**Figure 3.** (A) Determination of ras isoform expression in human neutrophils. Neutrophils were resuspended directly in 2× sample buffer, diluted, split equally between SDS-PAGE gels, and subjected to Western blotting. Gels were probed individually with isoform-specific antibodies. Lane 1 = 2 \(\times\) 10\(^{6}\) cells, lane 2 = 1 \(\times\) 10\(^{6}\) cells, lane 3 = 0.5 \(\times\) 10\(^{6}\) cells, lane 4 = 0.25 \(\times\) 10\(^{6}\) cells. Results are representative of three independent experiments. (B) Determination of isoform activation by ANCA. Neutrophils were primed with TNF-\(\alpha\), stimulated with 250 \(\mu\)g of ANCA for 10 min, and then subjected to the ras activation assay. After this, samples were split equally between SDS-PAGE gels and subjected to Western blotting. Gels were probed individually with isoform-specific antibodies or the pan-ras antibody (C, unstimulated neutrophils; A, ANCA-treated neutrophils). Blots were subsequently stripped and reprobed with anti-GST to indicate equal loading (right). Results are representative of three independent experiments using various combinations of neutrophil donors and ANCA preparations.

**Figure 4.** Effect of inhibition of phosphatidylinositol-3 kinase on ras activation by ANCA. Neutrophils were isolated and preincubated with either 50 \(\mu\)M LY294002 for 30 min at 37\(^{\circ}\)C or 10 nM Wortmannin for 5 min at 37\(^{\circ}\)C before priming with TNF-\(\alpha\) and incubation with 250 \(\mu\)g of ANCA or 167 \(\mu\)g of fraction antigen binding [F(ab\(^{\prime}\))\(_{2}\)] (molar equivalent) for 10 min at 37\(^{\circ}\)C. Subsequently, the ras activation assay was performed and samples were subjected to Western blotting as described. (A) Effects of LY294002. Lane 1, unstimulated neutrophils; lane 2, unstimulated neutrophils + LY294002; lane 3, ANCA-treated neutrophils; lane 4, ANCA-treated neutrophils + LY294002. (B) Effects of Wortmannin. Lane 1, unstimulated neutrophils; lane 2, unstimulated neutrophils + Wortmannin; lane 3, ANCA-treated neutrophils; lane 4, ANCA-treated neutrophils + Wortmannin. (C) Lane 1, unstimulated neutrophils; lane 2, unstimulated neutrophils + LY294002; lane 3, F(ab\(^{\prime}\))\(_{2}\)-treated neutrophils; lane 4, F(ab\(^{\prime}\))\(_{2}\)-treated neutrophils + LY294002. Blots were subsequently stripped and reprobed with anti-GST to indicate equal loading (bottom). Results are representative of three independent experiments using various combinations of neutrophil donors and ANCA preparations.
kine inhibitor) for 30 min or 200 ng/ml radicicol (selective inhibitor tyrosine kinases, e.g., Lyn) for 2.5 h before treatment with 250 μg of whole ANCA or equivalent F(ab’)2, followed by the ras activation assay. Neither inhibitor had any effect on viability (PP2 97.4 ± 1.15% or radicicol 98.2 ± 1.82%), total cellular p21ras (data not shown), or basal p21ras activity. Radicicol was unable to inhibit the p21ras stimulation seen in the presence of ANCA (data not shown), but PP2 gave a complete inhibition (Figure 5A). The inhibitory effects of PP2 extended to the stimulation seen in the presence of F(ab’)2 fragments alone (Figure 5B).

Discussion

We previously demonstrated that both whole ANCA IgG and its F(ab’)2 fragments are able to activate p21ras in neutrophils (5). This activation is inhibitable by both pertussis toxin and genistein, suggesting a dependence on G proteins as well as tyrosine kinases. In this investigation, we take these findings further and show that the activation of p21ras is transient and functionally linked to superoxide production, indicating a pivotal role for the GTPase. Upstream of the activation, p21ras is regulated in a dual manner by both PI3K and Src-family kinases. To the best of our knowledge, there was no evidence of the isoforms of p21ras expressed in human neutrophils, so we determined both the presence and the relative abundance of the different types. Moreover, we were able to demonstrate that there is a differential activation of the K-ras isoform by ANCA.

The extent and the duration of p21ras activation have been shown to be important factors in dictating the downstream events initiated by p21ras. Transient activation can lead to a proliferative response from the cell in question, whereas a more sustained level of active p21ras results in differentiation or senescence (6). In ANCA stimulation of neutrophils, we have shown a peak of activity at approximately 5 to 10 min, which returned to basal by 30 min. This transience could be directing the nature of the downstream intracellular events that take place (11). Neutrophils are terminally differentiated and therefore will not undergo proliferation in response to ANCA, but the time course of p21ras activity could influence which effectors can be activated successfully in the pathway. In these experiments, the increase in p21ras activity preceded the increase in superoxide production. It therefore was hypothesized that the GTPase may have a role in the generation of reactive oxygen species.

FTS is a specific and selective inhibitor of p21ras. The synthetic S-prenyl derivative of carboxylic acid affects the interaction of the small GTPase with the plasma membrane. Within 30 min of treatment, a large proportion of the p21ras is displaced and becomes degraded in the cytosol (12). The concentrations and incubation time used in these experiments had no effect on cell viability, which is confirmed by other researchers (13). In nontransformed cells, concentrations of 25 to 50 μM are required for inhibition (12). These concentrations have no effect on either Gβγ subunits (12) or NADPH oxidase (14) directly.

ANCA stimulation of p21ras was dose-dependently inhibited by FTS. This was paralleled by the inhibition of ANCA-induced superoxide production. It is important to note that p21ras is activated by F(ab’)2 fragments, but these alone are unable to induce a respiratory burst. This indicates that although p21ras is necessary for superoxide production in this system, other components are also required. Santillo et al. (15) also found that an inhibition of p21ras led to a decrease in superoxide production. However, this was shown in transfected cells to be due to H-ras rather than to K-ras. The overexpression of proteins such as p21ras is often used to investigate functional effects, but caution has to be used in the interpretation of such nonphysiologic systems. The activation of individual isoforms in nontransformed cells provides a more robust model for investigation.

For the first time, we have shown that only two isoforms of p21ras are present as protein in whole-cell lysates of human neutrophils. This novel finding is substantiated by findings from lymphocytes. Genot and Cantrell (16) found that K- and N-ras were the predominant isoforms in lymphocytes. It is of little surprise that K-ras is present as this is the most ubiquitously expressed of all of the isoforms, and knockouts have been shown to be embryonically lethal (7). N-ras is never expressed alone, and mutations in this isoform have been shown to be prevalent in myelomas (6). Lysis of whole cells does not indicate the localization of the two isoforms, which may be an important factor in effector activation and regulation of activity. Using semiquantitative approaches, we were able to show...
that there was more of the N isoform present than the K isoform. On the Western blots, doublets that illustrated that both prenylated (lower band) and nonprenylated (upper band) (17) forms were present in the neutrophils were also seen.

It is interesting that ANCA was able to activate only K-ras. This is intriguing given that this is the least expressed of the two isoforms. K-ras has been shown to be important in migration (18,19) and has a more pivotal role to play in the activation of Rac than the other isoforms (15). It is thought that the kinetics of GTP/GDP exchange on p21ras may vary between isoforms (18), and this therefore may influence the transient nature of the activation. K-ras is the least homologous of all of the isoforms with regard to posttranslational processing. It has a string of polybasic amino acids that enable it to interact with the plasma membrane in an electrostatic manner. This may confer an ability to interact with a defined subset of effectors, thus leading to a specific pattern of downstream events. p21ras is able to be activated by F(\(ab\))\(_2\) fragments via G proteins; this may also be a function of its charge in that it interacts with both acidic phospholipids and proteins (18) that could lead to microlocalization in areas of PR3 expression.

PI3K is both a lipid and a protein kinase whose main product is PIP3. We have previously determined that ANCA was not able to activate the p85/p110 isoform but did result in PI3K activity and PIP3 production (2). This indicated the likely involvement of the p101/p110\(\gamma\) isoform, which is regulated via G protein–coupled receptors. Using the inhibitors LY294002 and wortmannin (concordance of which is generally accepted as solid criterion for the involvement of PI3K), we have now shown that inhibition of PI3K leads to cessation of ANCA stimulation of p21ras. In addition, we have determined that this inhibition is through the F(\(ab\))\(_2\)–activated G protein pathway. Conventionally, PI3K is located downstream of p21ras activation, but a permissive role for the molecule has been confirmed in systems such as the G protein–coupled LPA receptor (20). Rubio and Wetzker (21) showed that basal Ras-GTP declined in U937 cells when PI3K was inhibited. This was determined to be a function of its charge in that it interacts with both acidic phospholipids and proteins (18) that could lead to microlocalization in areas of PR3 expression.

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PI3K is also able to interact directly with p21ras (21), and this may occur after ANCA stimulation. PI3K has been shown to interact with negatively charged phospholipids within the plasma membrane (26). This would place it in an ideal position to activate the positively charged K-ras. We cannot determine from our data whether it is the lipid kinase activity that is necessary for p21ras activation by PI3K or the protein kinase function.

We have also demonstrated that p21ras activation is dependent on Src-family kinases via the use of PP2. This inhibitor was able to decrease p21ras activation both by whole ANCA and by F(\(ab\))\(_2\), indicating a role for Src kinases in the G protein pathway, which is in addition to their recently described role in the tyrosine kinase pathway (4). p21ras activation may depend on basal (as opposed to stimulated) tyrosine kinase activity. Bar-Sagi and Hall (27) reported that a Src-family kinase lay downstream of G\(\beta\)\(\gamma\) subunits in the ras-activation pathway. They postulated that this gave rise to the instigation of the Shc and Sos complex, resulting in p21ras-GTP. This mechanism was also hypothesized by Schmitt and Stork (28) along with transactivation of the EGF receptor by G\(\beta\)\(\gamma\) subunits. Alternatively, v-Src is able to interact with PI3K directly (29), but this has not been demonstrated in G protein–coupled pathways. Radicicol failed to show any effects on p21ras stimulation with ANCA. This inhibitor has no effect on PI3K\(\gamma\) directly or the G\(\beta\)\(\gamma\) subunits (30) and is used as an inhibitor of the Lyn-stimulated p85 PI3K. As our previous work has negated a role for this isoform, one would not expect radicicol to have any effects.

In conclusion, we have shown that ANCA is able to activate p21ras in a time-dependent, isoform-selective manner that is reliant on initiation by Src and PI3K. The functional consequences of this activation include the production of superoxide by the neutrophils. This could provide a possible therapeutic opportunity for the treatment of ANCA-associated vasculitis. FTS has been shown in animal models to ameliorate certain dysregulated immune-mediated damage. For example, in a mouse model of experimental autoimmune encephalomyelitis, administration of FTS leads to a damping of the T cell response, specifically those that are targeting myelin, without a concomitant decrease in normal cell function (31). This is thought to occur through the selective action of FTS on cells with high levels of GTP-bound p21ras. This therefore could lead to its use as an immunosuppressant. In addition, the knowledge that ANCA initiates activation of only K-ras could give rise to the development of specific isoform blocking agents. These agents may circumvent the nonspecific effects seen with the majority of agents in clinical use for the treatment of vasculitis.

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