

## Protein Phosphatase 2A Regulates Apoptosis in Neutrophils by Dephosphorylating Both p38 MAPK and Its Substrate Caspase 3\*

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**The induction of apoptosis in neutrophils is an essential event in the resolution of an inflammatory process. We found recently that the reduction of the activity of the neutrophil survival factor p38 MAPK and dephosphorylation and thus activation of caspases must occur to initiate such cell death in these leukocytes. Here, we report a previously undetected early and transient activation of protein phosphatase 2A (PP2A) in neutrophils undergoing apoptosis. The pharmacological inhibition of this phosphatase during Fas-induced apoptosis augmented the levels of phosphorylation of both p38 MAPK and caspase 3, resulting in a decreased activity of caspase 3 and an increased neutrophil survival. The complementary finding of a time-dependent association among PP2A, p38 MAPK, and caspase 3 in intact neutrophils indicated that there is a direct regulatory link among these signaling enzymes during Fas-provoked apoptosis. Moreover, immunoprecipitated active p38 MAPK and recombinant phosphorylated caspase 3 were dephosphorylated by exposure to purified PP2A *in vitro*. Consequently, the early and temporary activation of PP2A in neutrophils impaired not only the p38 MAPK-mediated inhibition of caspase 3 but also restored the activity to caspase 3 that had already been phosphorylated and thereby inactivated. These findings indicate that PP2A plays a pivotal dual role in the induction of neutrophil apoptosis and therefore also in the resolution of inflammation.**

Human neutrophils are short-lived cells that undergo apoptosis soon after they are released from the bone marrow (1–3). The life span of these leukocytes is regulated by endogenous factors and extracellular stimuli, which in turn determine the length of an inflammatory process. Neutrophil apoptosis can be delayed by several factors in the local environment, such as lipopolysaccharides and granulocyte-macrophage colony-stimulating factor, whereas it is accelerated by Fas ligand (4, 5). Knowledge about the signaling mechanisms involved in the regulation of apoptosis in human neutrophils is still fairly limited, in part because of methodological limitations, that is, the short life span of neutrophils rules out transfection, and microinjection of these cells is not feasible (6).

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In previous studies, our research team (7) and other investigators (8) have observed that p38 mitogen-activated protein kinase (MAPK)<sup>1</sup> exhibits constitutive activity in freshly isolated human neutrophils, and we noted that this activity is transiently decreased and subsequently regained during spontaneous and Fas-induced apoptosis. Furthermore, we proposed that the constitutive activity of p38 MAPK in neutrophils is probably caused by factors present in the blood; therefore, the elimination of such elements during the isolation of these primary cells could explain the subsequent decrease in this activity. In the same study, we also found that the inactivation of the intrinsic p38 MAPK activity increased the number of apoptotic neutrophils. The mechanism underlying Fas-induced inactivation of p38 MAPK has not been identified. However, in a very recent study (9), we identified caspase 8 and 3 as downstream targets of p38 MAPK in neutrophils, and we found that p38 MAPK-induced phosphorylation of the serine 150 residue of caspase 3 and the serine 364 residue of caspase 8 impaired the activities of these caspases, thereby favoring survival of the neutrophils. These observations suggest that neutrophil apoptosis is initiated by and dependent on the inactivation of p38 MAPK.

The molecular mechanisms whereby extracellular factors modify the activity of p38 MAPK in neutrophils undergoing apoptosis are currently unknown. The serine/threonine kinase p38 MAPK is activated by the concomitant phosphorylation of tyrosine and threonine residues within a conserved threonine-glycine-tyrosine motif in its activation loop (10). Accordingly, p38 MAPK can be down-regulated by various protein phosphatases that dephosphorylate either the threonine or the tyrosine residue or both (11). One such enzyme is protein phosphatase type 2A (PP2A). PP2A is a phosphoserine/phosphothreonine phosphatase, which has been reported (12, 13) to dephosphorylate the threonine residue of p38 MAPK and thereby impair its activity. PP2A is also a highly conserved phosphatase that plays essential roles in several signal transduction pathways, translational control, endosome trafficking, cell cycle regulation, and apoptosis (14). The PP2A holoenzyme consists of a 36-kDa catalytic subunit C (C $\alpha$  or C $\beta$ ) and a 65-kDa structural subunit A (A $\alpha$  or A $\beta$ ), that together form the inseparable AC core dimer (PP2A<sub>AC</sub>). The A subunit appears to function primarily as a scaffolding structure that assembles the different subunits into the holoenzyme complex. PP2A also comprises a third highly diversified regulatory subunit B (B, B', or B'') that regulates the substrate specificity and subcellular localization of the holoenzyme complex (14). Theoretically, the various combinations of the subunits can give rise to a large number of different PP2A holoenzymes.

In the present study, we conducted experiments to deter-

<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; PP, protein phosphatase; Ab, antibody; Cal, calyculin; JNK, c-Jun NH<sub>2</sub>-terminal kinase; ATF, activating transcription factor.

mine whether PP2A is involved in the regulation of neutrophil apoptosis and thus also in the termination of inflammatory responses.

#### EXPERIMENTAL PROCEDURES

**Cells**—Human neutrophils were isolated under endotoxin-free conditions from whole blood drawn from healthy volunteers. The neutrophils were isolated by dextran sedimentation followed by a brief hypotonic lysis of contaminating erythrocytes and centrifugation on Ficoll-Paque (Amersham Biosciences) at 4 °C, as described originally (15). The isolated cells were immediately suspended at 4 °C in a complete RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 0.2 mM L-glutamine, and 100 µg/ml penicillin and streptomycin at a concentration of  $5 \times 10^6$  cells/ml. Cell aliquots for the time 0 values were taken immediately after the cells were suspended in this medium. The incubations of the cells were then initiated by placing them in multiwell cell culture plates at 37 °C in a humidified 5% CO<sub>2</sub> and 95% air environment. Fas receptors were engaged by incubating the cells with an anti-Fas monoclonal Ab (150 ng/ml) (catalog no. CH-11, Beckman) for the indicated periods of time.

**Immunoprecipitation and Western Blot Analysis**—Neutrophils were lysed in a buffer (if not otherwise indicated) containing 1% (v/v) Triton X-100, Tris 20 mM (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM β-glycerolphosphate, 2.5 mM sodium pyrophosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride after which the remaining cell debris was removed by centrifugation (7). The supernatant was precleared with protein G PLUS-agarose (Oncogene Science), after which one of the following was added: agarose-conjugated anti-phospho-p38 MAPK (Thr-180/Tyr-182) IgG<sub>1</sub> Ab (New England Biolabs), anti-p38 MAPK IgG Ab (Santa Cruz Biotechnology), or anti-caspase 3 polyclonal IgG Ab (Santa Cruz Biotechnology). As a control, one of the following Abs was added: agarose-conjugated anti-c-Myc IgG<sub>1</sub> (Clontech), anti-Fyn IgG<sub>1</sub>, non-immune anti-rabbit IgG (Santa Cruz Biotechnology), non-agarose-conjugated anti-PP2A IgG<sub>2b</sub> (clone 1D6) (Upstate Biotechnology) or anti-E-cadherin IgG<sub>2</sub>. The samples were subsequently incubated overnight under rotation at 4 °C. Non-agarose-conjugated immunocomplexes were allowed to absorb to protein G-agarose for 1 h at 4 °C. The immunoprecipitates were washed four times with lysis buffer, and either the precipitates listed above or lysates of intact cells were boiled in a sample buffer (7), after which the proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were analyzed with the following: anti-PP2A IgG<sub>2b</sub> Ab (clone 1D6) or monoclonal Ab 4G10 (mouse anti-phosphotyrosine) (Upstate Biotechnology); anti-caspase 3 IgG Ab (Santa Cruz Biotechnology), anti-p38 MAPKα IgG Ab, or anti-hemagglutinin IgG<sub>2a</sub> Ab (catalog no. F-7, Santa Cruz Biotechnology); anti-phospho-p38 MAPK (Thr-180/Tyr-182) IgG<sub>1</sub> Ab, anti-phospho-ATF2 (Thr-71) IgG Ab (New England Biolabs), or anti-phosphoserine IgM<sub>1</sub> Ab (catalog no. 16B4, Biomol). As indicated in the figure legends, certain blots were stripped and reprobed according to the instructions of the manufacturers.

**PP2A Phosphatase Assay**—The PP2A phosphatase assay was performed as stipulated by the manufacturer (serine/threonine assay kit 1, Upstate Biotechnology). Briefly, neutrophils were preincubated on ice for 15 min in the absence or presence of 3 µM PP1 (Biomol) or for 45 min with or without 20 µM DEVD-fluoromethyl ketone (ICN Biomedical, Inc.). Thereafter, the cells were exposed to anti-Fas Ab at 37 °C for different periods of time and were then lysed as indicated above in a lysis buffer (1% Nonidet P-40, Tris 50 mM (pH 7.5), 137 mM NaCl, 2 mM NaF, 10% (v/v) glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na<sub>3</sub>VO<sub>4</sub>). Subunit C (catalytic) of PP2A was immunoprecipitated as described above. The precipitates were washed by centrifugation twice in the lysis buffer and twice in a serine/threonine kinase buffer (100 µM CaCl<sub>2</sub> and 50 mM Tris-HCl (pH 7.0)). The pellet was resuspended in the kinase buffer, and PP2A activity was measured after the addition of 250 µM phosphopeptide. The phosphatase reactions were carried out for 15 min at 30 °C. The release of phosphate from the added phosphopeptide was quantified using a malachite green reagent. In short, a sample (25 µl) of the reaction medium was transferred to a microtiter assay plate, and 100 µl of malachite green reagent was added to each well, after which the plates were incubated for 15 min at 30 °C. Changes in absorbance were measured at 650 nm in a Fluostar plate reader (BMG Lab Technologies). The phosphatase activity in each well was determined as a percentage of the maximal activity recorded in each experiment. Alternatively, PP2A (10 units/ml) purified from human erythrocytes (Upstate Biotechnology)

was added to agarose-conjugated active (phosphorylated) p38 MAPK immunoprecipitates in the presence or absence of 10 nM calyculin A (Cal A). The phosphatase reactions were carried out at 30 °C for 30 min, and the activity remaining in the p38 MAPK immunoprecipitates was measured by running a p38 MAPK phosphorylation assay in the presence of ATF2-(19–96), as described previously (7).

**Expression of Human Caspase 3**—The pET21b vector containing C-terminally His<sub>6</sub>-tagged human pro-caspase 3 (16) was generously provided by Dr. E. S. Alnemri. Recombinant human caspase 3 was purified as described previously (9).

**In Vitro Assays for p38 MAPK Phosphorylation and PP2A Dephosphorylation**—The p38 MAPK phosphorylation assay was performed as reported previously (7, 9) in the presence of recombinant caspase 3 or active ATF2-(19–96). The assays were run for 60 min at 30 °C. Thereafter, radiolabeled phosphorylated caspase 3 was either incubated in the presence of 10 units/ml PP2A (Upstate Biotechnology) or was immunoprecipitated in active PP2A from neutrophils stimulated with anti-Fas Ab for 30 min, and a PP2A phosphatase assay was run as described above. The degree of phosphorylation of recombinant caspase 3 was analyzed by autoradiography, whereas the phosphorylation of ATF2-(19–96) was assessed by Western blotting (7).

**<sup>32</sup>P Phosphorylation of Caspase 3 in Vivo**—Neutrophils ( $5 \times 10^7$ /ml) were preincubated for 1 h and 50 min at 37 °C in a calcium- and phosphate-free medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 5.0 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 0.1% human bovine serum albumin, and 2 mM HEPES (pH 7.4)) supplemented with [<sup>32</sup>P]orthophosphate (2 mCi/ml). Thereafter, the cells were washed and resuspended in RPMI medium supplemented with 5% fetal calf serum, and some cells were also exposed to 40 nM Cal A for 10 min at 37 °C. Next, the cells were stimulated with the anti-Fas monoclonal Ab at 37 °C for 30 min in the absence or presence of 40 nM Cal A. Caspases were subsequently immunoprecipitated as described above using agarose-conjugated anti-caspase 3 polyclonal Ab. The degree of phosphorylation was analyzed by autoradiography performed on a PhosphorImager, and the membranes were subsequently immunoblotted for caspase 3.

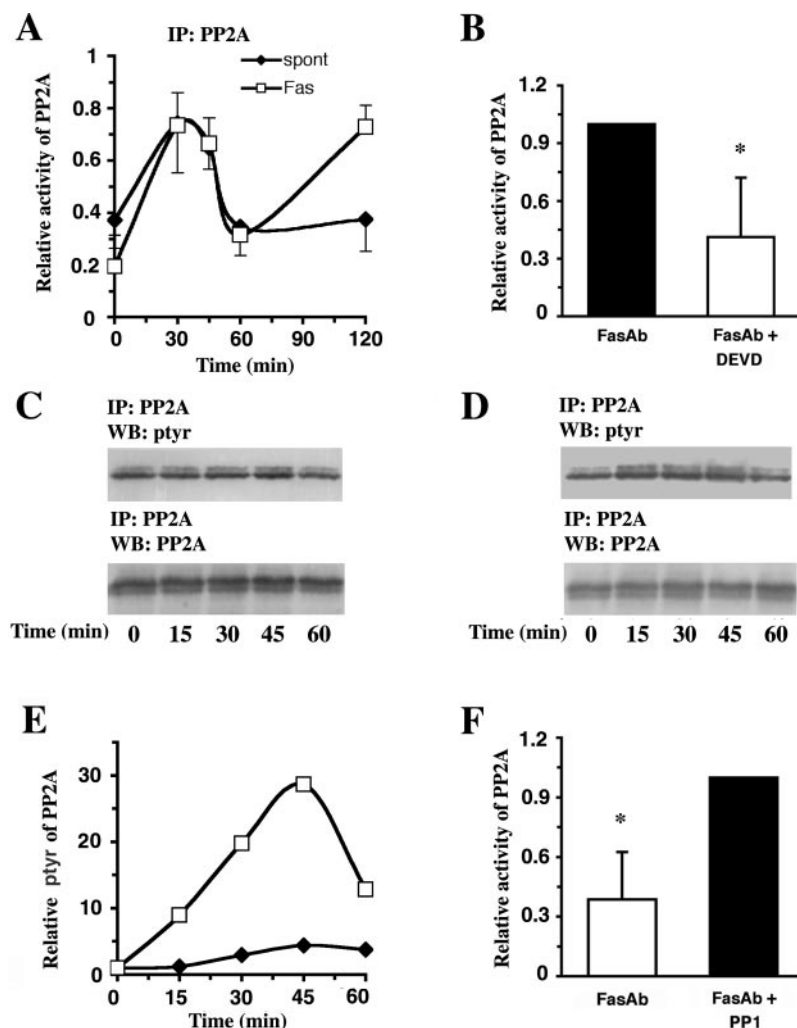
**Fluorometric Assays for Caspase Activities**—Neutrophils were first incubated in the absence or presence of 40 nM Cal A for 20 min on ice prior to exposure to anti-Fas Ab (150 ng/ml). The cells were incubated with the anti-Fas Ab for 75 min at 37 °C and then harvested and lysed as described previously (7). DEVD-7-amino-4-methylcoumarin (Upstate Biotechnology), which is a fluorogenic substrate for caspase 3, was added to each cell lysate, and the activity of caspase 3 (cleavage of 7-amino-4-methylcoumarin) was measured as described previously (7).

**Analysis of Nuclear Morphology**—Neutrophils were incubated for 15 min at 4 °C in the absence or presence of 40 nM Cal A. Thereafter, the cells were washed with RPMI 1640 medium at 4 °C prior to the engagement of their Fas receptor. The cells were incubated for 3 h at 37 °C in the presence of anti-Fas Ab. Subsequently, 25 µl of each cell suspension was transferred to an Eppendorf tube, and 1 µl of a mixture of acridine orange and ethidium bromide (100 µg/ml each) was added to the cell suspension. The stained cells were examined immediately in a Nikon Eclipse E800 fluorescence microscope equipped with a 4,6-diamidino-2-phenylindole/fluorescein isothiocyanate filter. A minimum of 100 nuclei was counted in each sample, and the percentage of nuclei displaying condensed chromatin was calculated.

**Statistical Evaluations**—All data are expressed as means ± S.D. ( $n < 6$ ) or S.E. ( $n \geq 6$ ), and Student's paired *t* test was used for statistical analysis of the differences.

#### RESULTS

**Activity and Regulation of the Serine/Threonine PP2A in Neutrophils Undergoing Apoptosis**—To address the possibility that a protein phosphatase regulates the previously demonstrated transient loss of p38 MAPK activity during Fas-induced apoptosis in human neutrophils (7), we measured the phosphatase activity in immunoprecipitates of PP2A (the catalytic subunit). Spectrophotometric analysis revealed a rapid increase in PP2A activity that reached a peak after 30–45 min and declined to the starting level after 1 h at 37 °C in the absence or the presence of the anti-Fas Ab (Fig. 1A). Notably, PP2A regained its activity after 2 h of Fas-induced apoptosis (Fig. 1A). This pattern of action correlated well with the increased activity of caspase 3 we recorded previously under the same conditions (9). Santoro *et al.* (17) have reported that caspase 3 can cleave and thereby activate PP2A during the execution phase of

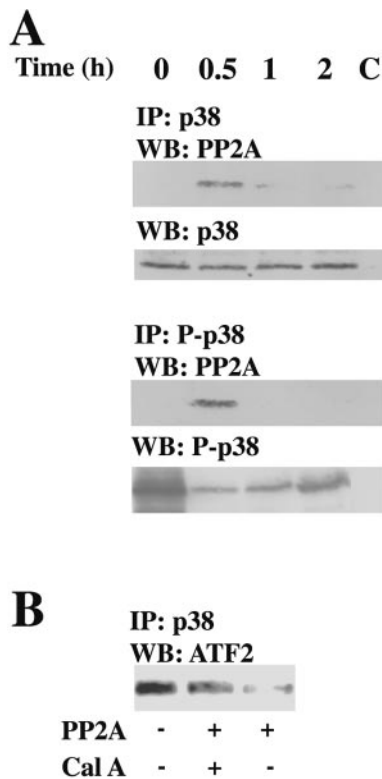


**FIG. 1. Activity and regulation of PP2A in neutrophils undergoing apoptosis.** **A**, neutrophils were incubated in the absence or presence of an anti-Fas Ab (150 ng/ml) for the indicated periods of time and then immediately lysed. Samples of the lysates were taken for immunoprecipitation (IP) with an anti-PP2A Ab, and the activity of PP2A in the immunoprecipitates was determined by incubation with a phosphopeptide substrate. The amount of phosphate that was released was quantified by recording changes in absorbance. PP2A phosphatase activity was expressed as the percentage of maximal activity in each experiment ( $n = 5-7$ ). Note that the increase in PP2A activity was more pronounced during Fas-induced apoptosis because it started from a lower initial level. **B**, neutrophils were preincubated for 45 min on ice in the presence or absence of 20  $\mu$ M DEVD-fluoromethyl ketone, and subsequently anti-Fas Ab was added, and the cells were incubated for 2 h and then lysed. PP2A phosphatase activity was expressed as the percentage of anti-Fas Ab-treated cells ( $n = 4$ ). PP2A immunoprecipitates from non-treated (**C**) or anti-Fas Ab-treated (**D**) cells were analyzed by Western blotting (WB) with mAb 4G10 (ptyr, mouse anti-phosphotyrosine) and then stripped and reprobed with an Ab against PP2A. The blots shown are representative of at least three separate experiments. **E**, the graph outlines the mean values of the densitometric analysis of the Western blotting probe for tyrosine phosphorylation of PP2A during spontaneous (**C**) and anti-Fas Ab-induced apoptosis (**D**). The degree of PP2A tyrosine phosphorylation was expressed as the percentage of the phosphorylation level found at time 0 in each experiment ( $n = 3$ ). **F**, neutrophils were preincubated for 15 min on ice with or without 3  $\mu$ M PP1. Thereafter, the cells were incubated for 45 min in the presence of anti-Fas Ab, lysed, and analyzed as in **A**. PP2A phosphatase activity in cells not treated with PP1 was expressed as the percentage of PP1-treated cells ( $n = 5$ ). \*,  $p < 0.05$ .

apoptosis. To determine whether the second increase in PP2A activity that we observed might have been caspase 3-dependent, we used DEVD-fluoromethyl ketone to inhibit the activity of this caspase. Such treatment decreased the activity in PP2A immunoprecipitates of Fas-treated cells after 2 h of incubation (Fig. 1B). These findings suggested that the second increase in PP2A activity we noticed (Fig. 1A) was caused by the Fas-induced increase in caspase activity in neutrophils. Therefore, we focused further experiments on elucidating the molecular mechanisms that regulated the transient activity of PP2A seen in Fas-treated cells during the first hour of incubation. The activity of PP2A can be modulated by different mechanisms, some of which involve the various regulatory subunits of this protein. In addition, the core subunit can undergo a number of biochemical modifications that deactivate PP2A; for example, some tyrosine kinases belonging to the Src kinase family can

phosphorylate PP2A on tyrosine 307 (18). We found that in neutrophils the level of tyrosine phosphorylation of the core subunit of PP2A was only modestly affected during spontaneous apoptosis (Fig. 1, C and E), whereas it was increased temporarily during the first 45 min of Fas-induced apoptosis (Fig. 1, D and E). A comparison of PP2A tyrosine phosphorylation (Fig. 1E) with PP2A activity (Fig. 1A) during Fas-induced apoptosis revealed a parallel increase during the first 30 min, after which the phosphatase activity started to decrease before there was a drop in the phosphorylation. Incubating the cells with the Src kinase inhibitor PP1 augmented the activity of PP2A 45 min after the initiation of anti-Fas-induced apoptosis (Fig. 1F). This finding indicated that a Src kinase participated in the decrease in PP2A activity seen after 30 min during anti-Fas-induced apoptosis (Fig. 1A). Furthermore, these data argue against the involvement of a Src kinase in the initiation

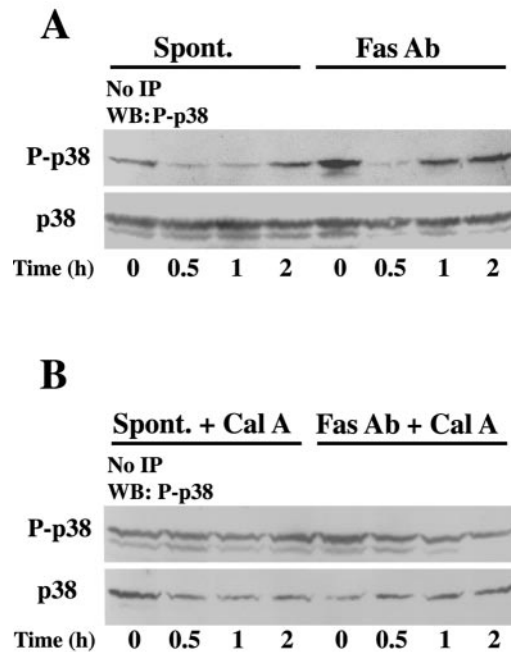




**FIG. 2. PP2A is co-immunoprecipitated with p38MAPK $\alpha$  and active P-p38 MAPK and affects the activity of p38 MAPK *in vitro*.** A, neutrophils were incubated at 37 °C with anti-Fas Ab for the indicated periods of time. Thereafter, the cells were lysed, and samples were immunoprecipitated (IP) with an anti-p38 $\alpha$ MAPK Ab (p38 $\alpha$ ), an anti-phospho-p38 MAPK Ab (P-p38), or an isotype-matched control Ab (C) and were subsequently assessed by Western blotting (WB). The blots were sequentially analyzed with the Abs against PP2A and then stripped and reprobed with either an Ab against p38 $\alpha$ MAPK (p38) or phospho-p38 MAPK (P-p38). The blots shown are representative of at least three separate experiments. B, freshly isolated neutrophils were lysed, and samples were immunoprecipitated with an anti-p38 $\alpha$ MAPK Ab (p38). Thereafter, the immunoprecipitates were incubated in the absence (–) or presence (+) of purified active PP2A (10 units/ml) and Cal A (10 nM) for 30 min at 30 °C. The activity of p38 MAPK in the immunoprecipitates was determined subsequently by incubation with ATP and a glutathione S-transferase-ATF2-(19–96) substrate, followed by Western blot analysis of phosphorylated ATF2-(19–96). The blot shown is representative of three separate experiments.

of PP2A activity during either spontaneous or Fas-induced apoptosis in neutrophils.

**PP2A Can Directly Regulate the Activity of p38 MAPK**—Our data showing corresponding activities of p38 MAPK (7) and PP2A suggested the existence of direct interactions between these signaling proteins, and that assumption was supported by the time-dependent recovery of PP2A from immunoprecipitates of total p38 $\alpha$  MAPK (the most abundant p38 MAPK isoform in neutrophils (19)) and active p38 MAPK from anti-Fas-treated cells (Fig. 2A). We observed that PP2A was not associated initially with p38 MAPK in freshly isolated neutrophils; instead, the association was first detected 30 min after the engagement of Fas receptors (Fig. 2A). To determine whether PP2A was involved in the Fas-induced dephosphorylation and inactivation of p38 MAPK, we used a precoupled antibody to immunoprecipitate total p38 $\alpha$  MAPK from freshly isolated neutrophils (because such cells exhibit a high level of p38 MAPK activity). These immunocomplexes were incubated in the absence or presence of active PP2A (10 units/ml) purified from human erythrocytes (Fig. 2B) for 30 min at 30 °C and were then washed extensively to remove residual PP2A. The

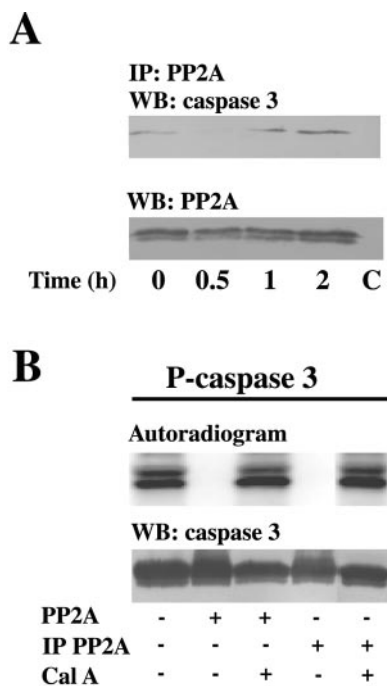


**FIG. 3. Inhibition of PP2A increases the phosphorylation/activity of p38 MAPK.** Neutrophils were incubated on ice in the absence (A) or presence (B) of 40 nM Cal A for 20 min and then at 37 °C with (Fas Ab) or without (Spont.) anti-Fas Ab for the indicated periods of time. The incubations were terminated by lysis of the cells, and samples were subsequently assessed by Western blotting (WB). The blots were sequentially analyzed with an Ab against active-p38 MAPK (P-p38) and then stripped and reprobed with an Ab against total p38 $\alpha$ MAPK (p38). The blots are representative of at least three separate experiments. IP, immunoprecipitation.

p38 MAPK activity that remained in these immunocomplexes was measured by performing an *in vitro* kinase assay for 1 h at 30 °C in the absence or presence of 10 nM Cal A with added ATF2. We detected a significant decrease in p38 MAPK-induced phosphorylation of ATF2 when immunoprecipitated p38 MAPK was incubated in the presence of PP2A (Fig. 2B). Interestingly, the PP2A-mediated decrease in p38 MAPK-induced phosphorylation of ATF2 was inhibited in the presence of Cal A (Fig. 2B). These results demonstrate that PP2A can directly dephosphorylate and inactivate p38 MAPK obtained from freshly isolated human neutrophils, which strongly suggests that PP2A is responsible for mediating the transient reduction of p38 MAPK activity seen in intact neutrophils.

**PP2A Causes the Transient Dephosphorylation of p38 MAPK during Apoptosis in Neutrophils**—Notably, the peak in PP2A activity (Fig. 1A) and the transient decline in p38 activity (Fig. 3A) (7) occurred simultaneously, and it was at the same point in time during an incubation that the activity of caspase 3 started to increase and could be detected (9). Considering the findings mentioned above, it is interesting that Cal A, which is an inhibitor of type 1 and 2A protein phosphatases (20), augmented the phosphorylation of p38 MAPK during the first hour of incubation and that the effect abolished the temporary decline in p38 MAPK activity (Fig. 3B). These observations support the idea that the initial, transitory inhibition of p38 MAPK during neutrophil apoptosis is caused by the transient increase in PP2A activity.

**PP2A Dephosphorylates Caspase 3 at Serine 150**—We had observed previously that p38 MAPK plays a role in the survival of human neutrophils by virtue of its ability to phosphorylate (at Ser-150) and thereby inactivates caspase 3 (9). That finding, along with the present results showing that PP2A participates in the regulation of p38 MAPK activity in neutrophils,



**FIG. 4. Caspase 3 is recovered from PP2A immunoprecipitates, and PP2A can dephosphorylate caspase 3 at Ser-150 *in vitro*.** *A*, neutrophils were incubated in the presence of an anti-Fas Ab for the indicated periods of time and then lysed, and samples were immunoprecipitated (IP) with an anti-PP2A Ab or an isotype-matched control (C) Ab. The immunoprecipitates were sequentially analyzed by Western blotting (WB) with an Ab against anti-caspase 3 and then stripped and reprobed with an Ab against anti-PP2A. The blots shown are representative of at least three separate experiments. *B*, phosphorylated recombinant caspase 3 (P-caspase 3) was incubated in the absence or presence of purified active PP2A (PP2A) or active PP2A immunoprecipitated from neutrophils (IP PP2A) that had been incubated for 30 min with an anti-Fas Ab. Cal A (10 nM) was added to the assay as indicated. Thereafter, samples incubated under the different experimental conditions were subjected to autoradiographic analysis of the phosphorylation level of caspase 3 and to Western blotting to ensure that equal amounts of phosphorylated caspase 3 had been added in each experiment.

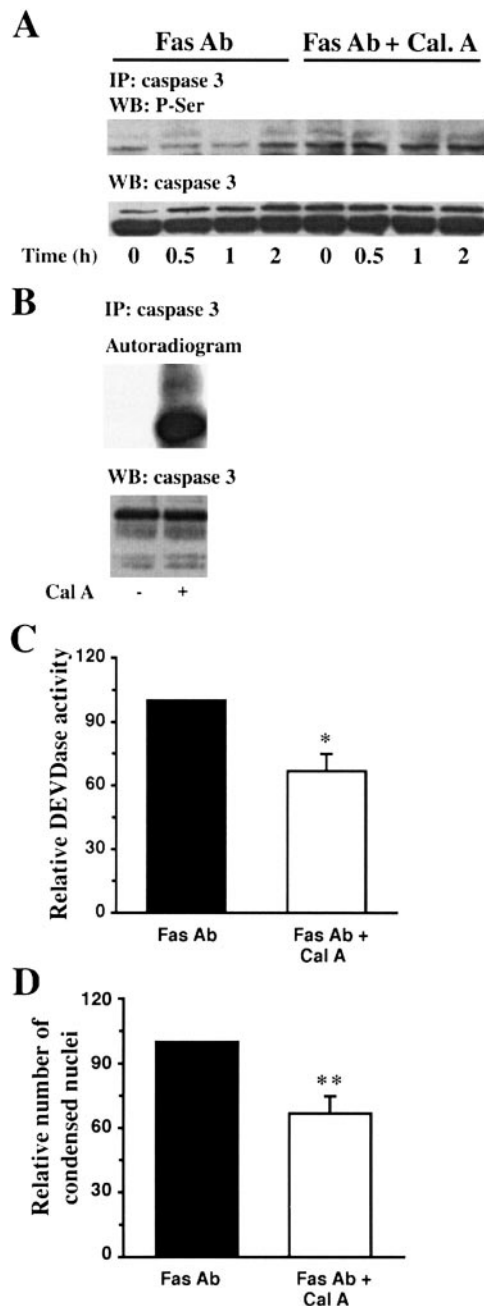
prompted us to determine whether PP2A also affects the activity of caspase 3 in neutrophils undergoing apoptosis. Caspase 3 has been found to cleave PP2A in Jurkat cells (17), which suggests that these two enzymes could also interact with each other in human neutrophils. Indeed, immunoprecipitation of caspase 3 and PP2A indicated a similar time-dependent interaction between these proteins in neutrophils (Fig. 4A), which suggests that PP2A can directly dephosphorylate caspase 3 and thereby regain its impaired activity. To test that assumption, we performed an assay *in vitro* to determine whether p38 MAPK-phosphorylated caspase 3 can serve as a substrate for PP2A. Active p38 MAPK that had been immunoprecipitated from newly isolated neutrophils was incubated with recombinant human caspase 3 in the presence of 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP (9). After p38 MAPK-dependent phosphorylation of caspase 3 had occurred, the agarose-conjugated active p38 MAPK immunoprecipitates were removed. The phosphorylated caspase 3 was incubated with 10 units/ml PP2A for 30 min at 30 °C and was purified of human erythrocytes and PP2A immunoprecipitates from neutrophils that had been exposed to anti-Fas Ab for 30 min in the presence or absence of 10 nM Cal A (Fig. 4B). Thereafter, we analyzed the level of caspase 3 phosphorylation by autoradiography and found that both purified and immunoprecipitated PP2A caused dephosphorylation of caspase 3 and that this dephosphorylation could be inhibited by Cal A (Fig. 4B). Together, these results indicate that PP2A can mediate Fas-induced dephosphorylation of caspase 3.

**PP2A Participates in Regulation of Neutrophil Apoptosis—** The interaction among p38 MAPK, caspase 3, and PP2A in intact neutrophils and our finding that PP2A can directly dephosphorylate p38 MAPK and caspase 3 *in vitro* suggest that PP2A plays an important role in the regulation of neutrophil apoptosis by directly inducing biochemical changes in both p38 MAPK and caspase 3, resulting in the reduced activity of p38 MAPK but the increased activity of caspase 3. To ascertain the validity of that assumption, we used two different experimental approaches to detect PP2A-dependent dephosphorylation of caspase 3 in intact neutrophils. First, we employed a general anti-phosphoserine antibody to investigate the effect of the PP2A inhibitor Cal A (40 nM) on the level of phosphorylation of immunoprecipitated caspase 3. We chose that method because it enables analysis of the phosphorylation immediately after isolation of the cells. The results show that the inhibition of PP2A in Fas-engaged neutrophils led to increased phosphorylation of caspase 3 (Fig. 5A). Second, we labeled freshly isolated human neutrophils with  $^{32}$ P for 1 h and 50 min and then incubated them for 10 min with or without 40 nM Cal A. We then engaged the Fas receptors of these cells in the absence or presence of Cal A. The time points indicated in Fig. 5B cannot be compared with those in the other figures because the cells in this experiment had been preincubated for a significant period of time at 37 °C before they were exposed to the anti-Fas Ab. Compared with Fas-treated cells,  $^{32}$ P-labeled cells incubated with anti-Fas Ab and Cal A displayed augmented phosphorylation of both the 32-kDa proform and the 20-kDa active form of caspase 3. To ascertain whether PP2A actually is involved in neutrophil apoptosis, we pretreated those leukocytes with Cal A and then analyzed the effect that this phosphatase inhibitor had on Fas-induced activation of caspase 3. The results revealed that the inhibition of PP2A significantly impeded the Fas-provoked activity of caspase 3 (Fig. 5C). In addition, we also analyzed the effect of Cal A on apoptosis by staining the nuclei of anti-Fas-treated neutrophils with acridine orange (Fig. 5D). These experiments show conclusively that the inhibition of PP2A decreased the number of anti-Fas Ab-treated cells with an apoptotic morphology. Together, these findings show that the inhibition of PP2A in Fas-engaged neutrophils led to an increased phosphorylation of caspase 3, which inhibited the activity of this caspase and thereby delayed the apoptotic process.

## DISCUSSION

We have shown previously that the ability of p38 MAPK to phosphorylate caspases constitutes a survival signal in isolated human granulocytes (7, 9). Specifically, such biochemical modifications impair the activities of caspases and thereby weaken the capacity of these proteins to induce apoptosis (9). Therefore, inactivation of the constitutively active p38 MAPK facilitates stimulation of the caspase cascade during spontaneous and Fas-induced neutrophil apoptosis (7). The mechanism of the temporary deactivation of p38 MAPK has not yet been examined in human neutrophils. In the present study, we noted that a transient increase in PP2A activity occurred at the same time as the decrease in p38 MAPK activity, which suggests that PP2A could be involved in regulating the phosphorylation and thus also the activity of p38 MAPK. The finding that both the increase in PP2A activity and the decrease in p38 MAPK activity take place at a point in time when caspase 3 starts to display increased activity (7, 9) implies that p38 MAPK as well as PP2A is involved in the control of neutrophil apoptosis.

The existence of a possible regulatory relationship between p38 MAPK and PP2A is supported by results reported by Keyse (11), showing that various protein phosphatases can down-regulate the activity of p38 MAPK by dephosphorylating either



**FIG. 5. Inhibition of PP2A in intact neutrophils increases the Fas-induced phosphorylation and decreases the Fas-induced activation of caspase 3.** *A*, neutrophils were incubated with an anti-Fas Ab in the absence or presence of 40 nM Cal A for the indicated periods of time and then lysed. Samples of the lysates were taken for immunoprecipitation (IP) of caspase 3, and the immunoprecipitates were sequentially analyzed with an Ab against anti-phosphoserine (P-Ser) and then stripped and reprobed with an Ab against anti-caspase 3. *B*,  $^{32}$ P-labeled neutrophils were incubated with an anti-Fas Ab for 30 min and then lysed. Caspase 3 immunoprecipitates of the lysates were subjected to gel electrophoresis, and the separated proteins were subsequently transferred to nitrocellulose membranes. The membranes were first analyzed with a PhosphorImager and then by Western blotting (WB) with an anti-caspase 3 Ab. The blots and autoradiographs illustrated in *A* and *B* are representative of at least three separate experiments. *C*, neutrophils were incubated on ice in the absence or presence of 40 nM Cal A for 15 min and then for an additional 1 h at 37 °C in the presence of anti-Fas Ab. Thereafter, the cells were lysed, and samples of the lysates were analyzed for DEVDase (caspase 3) activity ( $n = 6$ ). To adjust for differences between blood batches, the caspase activity in samples from cells not incubated with Cal A was defined as 100% in each separate experiment. *D*, neutrophils were incubated on ice in the absence or presence of 40 nM Cal A for 15 min and then, after removing Cal A, for an additional 3 h at 37 °C in the presence of anti-Fas Ab. The cells were subsequently stained with

a specific threonine or a specific tyrosine residue or both. The author documented that the serine/threonine phosphatases PP2A and PP2C $\alpha$  can deactivate p38 MAPK by phospho-threonine dephosphorylation (11). The present finding of a Fas-dependent association between PP2A and p38 MAPK provides the basis for a signaling interaction between these two enzymes and hence implies that PP2A is involved in regulating the induction of apoptosis in neutrophils.

To ascertain whether PP2A does play a role in neutrophil apoptosis, we pretreated such leukocytes with the phosphatase inhibitor Cal A, which is known to efficiently block the effects of PP1 and PP2A but not PP2C $\alpha$  (20). Only PP2A and PP2C $\alpha$  can dephosphorylate p38 MAPK, and therefore it seems reasonable to assume that any influence of Cal A on Fas-induced neutrophil apoptosis is mediated by its effect on PP2A. We found that the inhibition of PP2A by Cal A abolished the Fas-induced transient decrease in p38 MAPK activity and that it also increased the phosphorylation of caspase 3 and thereby reduced the activity of this apoptotic protease and the morphological alterations characteristic of apoptotic cells. Our data reveal an association between p38 MAPK and caspase 3 and show that PP2A interacts with both of those proteins. Consequently, it could be hypothesized that PP2A may directly dephosphorylate not only p38 MAPK but also caspase 3. In accordance with that idea, the results of our experiments performed *in vitro* revealed such a direct effect of PP2A on caspase 3 and on p38 MAPK, and therefore we propose that, in addition to its influence on p38 MAPK, PP2A has a direct impact on the phosphorylation statuses and activity of caspase 3. These findings suggest that PP2A promotes neutrophil apoptosis through dual actions, more precisely, by dephosphorylating p38 MAPK and thus inhibiting subsequent phosphorylation of caspase 3 and by dephosphorylating caspase 3 that has already been phosphorylated and thereby restoring its activity.

The signaling events that occur during the first hour of Fas-induced apoptosis in neutrophils are relatively straightforward to interpret, whereas the subsequent alterations in intracellular signaling are more difficult to understand because of the increased activity of caspases in apoptotic cells. The stimulated caspases are known to degrade and thereby abolish the activities of a number of signaling molecules (21), or, as in the case of PP2A, they increase its activity (17). We found that the inhibition of caspase 3 in neutrophils exposed to anti-Fas Ab for 90 min led to reduced activity of PP2A. Accordingly, it is logical to conclude that the secondary increase in PP2A activity observed in neutrophils with engaged Fas receptors is mediated indirectly by the Fas-induced increase in caspase activities. Based on this knowledge, we focused our present efforts on understanding how PP2A participates in the initial induction phase of apoptosis in neutrophils.

PP2A has been implicated primarily as a proapoptotic signal in several human and murine cell lines (22–24), and such an effect is mediated probably by the ability of this protein to dephosphorylate Bcl-2 and Bad (22, 23). However, PP2A can also indirectly exert a proapoptotic effect on cells by down-regulating signal transduction molecules such as extracellular signal-regulated kinase (24). In tumor necrosis factor- $\alpha$ -stimulated human neutrophils, Avdi *et al.* (25) have demonstrated a functional cross-talk between p38 MAPK and JNK, whereby p38 MAPK acts to limit the activation of a proapoptotic signal

acridine orange to assess their nuclear morphology. To adjust for differences between blood batches, the number of neutrophils with condensed nuclei in samples from cells not incubated with Cal A was defined as 100% ( $n = 6$ ) in each separate experiment. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



from JNK. These authors suggest that this cross-talk is mediated via the p38 MAPK-mediated activation of PP2A and the subsequent inhibition of MAPK kinase 4 and JNK (25). In contrast, results from the present study suggest that the proapoptotic impact of PP2A in primary human neutrophils occurs through direct regulation of another member of the MAPK family, namely p38 MAPK, and also caspase 3, which is a downstream substrate of p38 MAPK. These effects are possible because of the interactions of PP2A with p38 MAPK and caspase 3, which enable a transient PP2A-mediated deactivation of p38 MAPK and the dephosphorylation and reconstitution of previously impaired caspase 3 activity. These molecular modifications, in turn, increase the activity of caspase 3 and thus initiate apoptosis in human neutrophils. Taken together, these data could of course be readily explained by the ability of Fas- and tumor necrosis factor- $\alpha$  receptors to activate distinctly different downstream signaling events that result in an increased neutrophil survival. However, the data convincingly reveal the capacity of both PP2A and p38 MAPK to regulate each other and show that such signaling interactions are important in regulating neutrophil survival.

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**Protein Phosphatase 2A Regulates Apoptosis in Neutrophils by Dephosphorylating  
Both p38 MAPK and Its Substrate Caspase 3**

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