Regulation of Acidification and Apoptosis by SHP-1 and Bcl-2

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Recruitment of the SH2 domain containing cytoplasmic protein-tyrosine phosphatase SHP-1 to the membrane by somatostatin (SST) is an early event in its antiproliferative signaling that induces intracellular acidification-dependent apoptosis in breast cancer cells. Fas ligation also induces acidification-dependent apoptosis in a manner requiring the presence of SHP-1 at the membrane. Moreover, we have recently reported that SHP-1 is required not only for acidification, but also for apoptotic events that follow acidification (Thangaraju, M., Sharma, K., Liu, D., Shen, S. H., and Srikant, C. B. (1999) Cancer Res. 59, 1649–1654). Here we show that ectopically expressed SHP-1 was predominantly membrane-associated and amplified the cytoxic signaling initiated upon SST receptor activation and Fas ligation. The catalytically inactive mutant of SHP-1 (SHP-1C455S) abolished the ability of the SST agonists to signal apoptosis by preventing the recruitment of wild type SHP-1 to the membrane. Overexpression of the anti-apoptotic protein Bcl-2 in MCF-7 cells inhibited SST-induced apoptosis upstream of acidification by inhibiting p53-dependent induction of Bax as well as by raising the resting pH and attenuating SST-induced decrease in pH. By contrast, Bel-2 failed to prevent apoptosis triggered by direct acidification. These data demonstrate that (i) membrane-associated SHP-1 is required for receptor-mediated cytoxic signaling that causes intracellular acidification and apoptosis, and (ii) Bcl-2 acts distal to SHP-1 and p53 to prevent SST-induced acidification but cannot inhibit the apoptotic events that ensue intracellular acidification.

Apoptosis is a unique physiological mechanism that eliminates discrete cells in normal development, host defense, and suppression of oncogenesis. Nuclear changes such as nuclear shrinking, chromatin condensation, oligonucleosomal DNA degradation into multimers of ~180 base pairs, and characteristic cleavage of nuclear proteins are principal and easily detected end points of apoptosis. The onset of nuclear catastrophe is preceded by events in non-nuclear regions of the cell including activation of proteases and ion-fluxes, and disruption of membrane potentials and cytoskeletal architecture. These are in turn regulated by other cellular components including protein kinases and phosphatases, and members of the Bcl-2 family.

The non-transmembrane tyrosine phosphatase SHP-1 (SHPTP1/PTP1C/HCP/SHP/PTP-N6) and SHP-2 (PTP1D/PTP2C/SHPTP2/Syk/sycorscrew) are critical regulators of cellular function. These protein-tyrosine phosphatases (PTP) contain two amino-terminal Src homology 2 homology domains (SH2) domains, a catalytic domain and a carboxyl-terminal regulatory domain (1–4). Despite sharing significant sequence identity, these two phosphatases are distinct in their biological roles. For instance, tyrosine kinase signaling is inhibited by SHP-1 but enhanced by SHP-2 (2, 5). SHP-1 is known to associate with multiple signaling molecules such as growth factor receptor tyrosine kinases, non-receptor tyrosine receptor, interleukin-3 receptor β chain, erythropoietin receptor, interferon α/β receptor, and FGFR1B receptor (6–12). Such an association allows SHP-1 to dephosphorylate and inactivate both receptor kinases and non-receptor tyrosine kinases such as Jak-2 (9, 10). In addition, SHP-1-mediated tyrosine dephosphorylation is implicated in the signaling of cellular apoptosis. Lymphoid cell apoptosis requires SHP-1 and is potentiated by its overexpression (13). Loss of SHP-1 expression in the mouse is reported to abrogate Fas-mediated lymphocyte apoptosis, although this finding has been questioned (14, 15).

Functional activation of PTP has been implicated in antiproliferative signaling mediated directly via G protein-coupled receptors that bind somatostatin (SST), angiotensin II, and dopamine (16–19). While early studies claimed that SST activates membrane-associated PTP directly we could not confirm such a finding (16, 17, 20). Instead, our analysis led to the observation that SST promotes translocation of PTP from the cytosol to the membrane (21). We and others have shown that the antiproliferative signaling of SST results in apoptosis in tumor cells derived from the breast (MCF-7, T47D) and pituitary (AtT-20) (22–24). Its cytotoxic action induces an increase in wild type (wt) p53 and Bax and leads to a decrease in pH, and apoptosis in MCF-7 and T47D cells (21, 24, 25). The PTP inhibitor orthovanadate completely inhibited the cytotoxic activity of SST (21, 24). We demonstrated that the cytotoxic signaling of SST is SHP-1-mediated (21). Direct acidification...
cause by pH clamping of the medium with the proton ionophore nigericin or by inhibiting Na/H exchanger (NHE) and H\textsuperscript{-}/ATPase also caused apoptosis in a SHP-1-dependent manner in MCF-7 cells (25). Moreover, SHP-1 was required not only for SST-induced intracellular acidification but also for acidification-triggered apoptosis to occur (25).

The mammalian anti-apoptotic protein Bcl-2 inhibits the collapse of mitochondrial inner transmembrane potential (\(\Delta \psi_m\)) and release of cytochrome c from the mitochondria (independent of \(\Delta \psi_m\)), events that are considered early features of apoptosis (26–30). Bcl-2, a membrane protein by virtue of its hydrophobic carboxyl-terminal membrane insertion domain (31), has no established enzymatic activity. Nevertheless, overexpression of Bcl-2 can rescue various types of cells from mitochondrial and nuclear manifestations of apoptosis (32–36). Little is known about how and at what point Bcl-2 protects against stimuli that promote intracellular acidification during apoptosis except that it may act upstream of acidification, possibly by delaying the decrease in \(pH_i\) (37, 38).

In order to obtain definitive evidence for the involvement of SHP-1 in SSTR-initiated and Fas-mediated intracellular acidification cytotoxic signaling, we evaluated the effect of the octapeptide SST analog octreotide (OCT) and Fas ligation in MCF-7 cells transfected with SHP-1 or its catalytically inactive mutant SHP-1C455S. Additionally, we investigated the effect of Bcl-2 overexpression in MCF-7 cells on apoptosis triggered by SST in order to determine at what point Bcl-2 can prevent SHP-1-mediated, acidification-dependent apoptosis signaled by SST. We report here that cytosolic acidification and apoptosis triggered by SST activation and Fas ligation are signaled through membrane-associated SHP-1. We also demonstrate that overexpression of Bcl-2 in MCF-7 cells attenuates the inductive effect of SST on Bax and intracellular acidification without influencing the ability of SST to recruit cytosolic SHP-1 to the membrane or to induce p53. However, Bcl-2 failed to prevent apoptosis triggered by direct acidification. These data indicate that (i) membrane-associated SHP-1 is required for intracellular acidification and apoptosis to occur and (ii) Bcl-2 exerts its cytoprotective effect upstream of acidification, but downstream of SHP-1 and p53, by inhibiting p53-dependent induction of Bax as well as by promoting intracellular alkalization and attenuating SST-induced decrease in \(pH_i\).

MATERIALS AND METHODS

MCF-7, human breast adenocarcinoma cell line (HTB 22), was obtained from ATCC, Bethesda, MD. Octreotide (OCT, SMI 201–995) and its tyrosinated analog [Tyr\textsuperscript{3}]OCT were obtained from Sandoz (Basel, Switzerland); v-[Trp\textsuperscript{8}]SST-14 was supplied by Bachem (Torrance, CA). p-Nitrophenyl phosphate (pNPP) was purchased from Sigma. Annexin V-FLUOS apoptosis detection kit and anti-CD-95 antibody were purchased from Roche Diagnostics Canada (Montreal, Quebec). Anti-p53 and anti-Bax antibodies were obtained from Oncogene Sciences (Cambridge, MA) and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. All other chemicals used were of analytical grade and were obtained from regular commercial sources.

Cell Culture and Transfections—Cells were plated in 75-cm\textsuperscript{2} culture flasks and grown to confluence in minimum essential medium containing 10% fetal bovine serum and 10 mg/ml \(\text{bovine insulin}\). Native SHP-1 and its catalytically inactive mutant with a Cys\textsuperscript{455} \(\rightarrow\) Ser mutation (SHP-1C455S) were constructed in the expression vector pRc/ctyomegalovirus as described previously (39). MCF-7 cells were transfected with 10 mg of the pRc/ctyomegalovirus vectors containing the respective cDNA by the Lipofectin method. Cells expressing the vectors were selected in medium containing 400 \(\mu\text{g}\) of G418. Cells thus selected from three independent transfections with the expression vector pRc/cytomegalovirus as described previously (24).

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FIG. 1. Overexpression of SHP-1 and SHP1C455S in MCF-7 cells. Top, whole cell extracts (25 \(\mu\text{g}\)) from control and transfected cells were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with anti-SHP-1 antisera. Bottom, cell extracts were immunoprecipitated with anti-SHP-1 antisera and the immunoprecipitates were analyzed for PTP activity using pNPP as the substrate.

PTP Assay and SHP-1 Immunoblotting—To assess the effect of treatment of cells with apoptotic stimuli on the cellular distribution of phosphatase activity and SST-1, flasks containing equal number of cells (5 \(\times\) 10\textsuperscript{4}) were incubated for 1 h in the absence or presence of 100 nM OCT, v-[Trp\textsuperscript{8}]SST-14, or anti-CD-95 antibody and cytosolic and membrane fractions prepared as described previously. To assess the effect of SST on translocation of SHP-1, flasks containing equal numbers of cells (5 \(\times\) 10\textsuperscript{4}) were incubated for 1 h in the absence or presence of 100 nM v-[Trp\textsuperscript{8}]SST-14. Cells were washed in PBS and resuspended in buffer containing 200 \(\mu\text{M}\) mannitol, 68 \(\mu\text{M}\) sucrose, 50 \(\mu\text{M}\) HEPES-KOH, pH 7.4, 50 \(\mu\text{M}\) KCl, 5 \(\mu\text{M}\) EGTA, 2 \(\mu\text{M}\) MgCl\textsubscript{2}, 1 \(\mu\text{M}\) dithiothreitol, and protease inhibitors. After incubation for 30 min on ice, the cells were homogenized by hand in a Dounce homogenizer with a glass pestle. Homogenates were centrifuged at 14,000 \(\times\) 8 g for 20 min and the supernatant and the pelleted membrane fractions were kept at \(-80^\circ\text{C}\). Phosphatase activity in membrane and cytosolic fractions was determined using pNPP as the substrate and SHP-1 immunoblot analysis were carried out as described previously (21, 25). The enzyme activity was 3-fold higher in SHP-1 expressing cells compared with untransfected MCF-7 cells (Fig. 1). In cells expressing the catalytically inactive mutant SHP-1C455S, despite the comparable increase in protein expression no increase in PTP activity was observed. To assess the effect of SST on translocation of SHP-1, flasks containing 5 \(\times\) 10\textsuperscript{5} cells were incubated for 1 h with or without 100 nM SST agonists and cytosolic and membrane fractions prepared and subjected to immunoblot analysis as described previously (21, 25).

Octreotide Binding to SSTR—\([\text{I}^3\text{H}-\text{Tyr}^3]\)OCT was iodinated by the chloramine-T method using carrier-free Na\textsuperscript{[3]}\text{I} and purified by reverse phase high performance liquid chromatography on a \(\mu\)-Bondapak column (21). The specific activity of the purified \([\text{I}^3\text{H}-\text{Tyr}^3]\)OCT was 2000 Ci/mmol. Competitive binding assays were carried out using cell membranes. Thirty nanomoles of the radioligand were incubated in the absence or presence of 0–100 nm unlabeled peptide with 50 \(\mu\text{g}\) of membrane protein at 30 \(^\circ\text{C}\) for 30 min in 50 nm HEPES-KOH buffer, pH 7.5, containing 5 nm Mg\textsuperscript{2+}, 0.02% bovine serum albumin, 200 kallikrein inhibitory units of aprotinin, and 0.02 \(\mu\text{g}\)/ml each of bacitracin and phenylmethylsulfonyl fluoride. The membrane associated radioactivity was separated by centrifugation, washed, and quantitated in a \(\gamma\)-spectrometer. The data were analyzed by the computer-assisted nonlinear regression analysis (Ligand) program.

Measurement of cAMP—In addition to its effect on SHP-1, SST affects other second messenger pathways including inhibition of the stimulated adenylyl cyclase-CAMP pathway (41). To determine if the ability of SST to negatively regulate the adenylyl cyclase-CAMP pathway in MCF-7 cells is influenced by expression of SHP-1 or its inactive mutant, we incubated the cells sequentially for 15-min intervals in 1 ml of medium containing 0.5 mM isobutyl-1-methylxanthine and in 1 ml of the same medium with or without 1 \(\mu\text{M}\) forskolin in the presence or absence of 100 nM v-[Trp\textsuperscript{8}]SST-14. The cells were then washed in phosphate-buffered saline and sonicated and extracted in 1 ml of 0.1 \(\times\) HCl. cAMP was measured by using a commercial radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA).
Detection of Apoptosis by Annexin-V Labeling and DNA Fragmentation Analysis—Following treatment with the peptide, cells were incubated with FITC-conjugated Annexin-V and PI using the apoptosis detection kit according to the manufacturer's instructions. Cellular fluorescence was excited by a 5-watt argon laser generating light at 488 nm. FITC emission was detected through a 515-nm long pass filter and FITC fluorescence was detected with a 560-nm short pass dichroic filter. At least 10,000 gated events were recorded for each sample and the data analyzed by Winlist software (Verity Software House, Topsham, ME). To assess DNA fragmentation, cellular DNA was extracted twice with phenol/chloroform and once with chloroform from cells incubated in lysis buffer (500 mM Tris-HCl, pH 9.0, containing 2 mM EDTA, 10 mM NaCl, 1% SDS, and 1 mg/ml protease K) at 48 °C for 30 h. DNA extracts were incubated with 300 mg/ml bovine pancreatic RNase A at 37 °C for 1 h and 10-μg aliquots of DNA samples containing 10 mg/ml ethidium bromide were subjected to inversion field gel electrophoresis on 1.2% (w/v) agarose gels using the Hoefer Switchback pulse controller and visualized under UV light.

Measurement of Intracellular pH—For measuring intracellular pH, cells were loaded with 10 μmol acetoxymethylester derivative of SNARF-1 during the final hour of incubation in the absence or presence of 100 nM SST agonists or 25 ng of anti-CD95 antibody at 37 °C (42). The cells were then scraped, washed, and maintained at 37 °C in a Becton-Dickinson FACStar Vantage cytometer. Intracellular carboxyl SNARF-1 fluorescence was excited at 488 nm and emission was recorded at both 580 and 640 nm with 5-nm band-pass filters with linear amplifiers. The ratio of the emissions at these wavelengths was electronically calculated and used as a parameter indicative of intracellular pH. The intracellular pH values in control and treated cells were estimated by comparison of the mean ratio of the samples to a calibration curve of intracellular pH generated by incubation of carboxy-SNARF-1-loaded cells in buffers ranging in pH from 8.0 to 6.25 and containing nigericin. Cells with fluorescent of <50 units were excluded in the calculation of the ratio of the emissions at 580 and 640 nm.

Measurement of p53 and Bax—Cells stained with anti-p53 or anti-Bax antibodies were counterstained with FITC-conjugated secondary antibody following which DNA was labeled with PI and the RNA removed by digestion with RNase I as described previously (24, 25, 43, 44). FITC and PI fluorescence emissions were measured as described above for apoptosis measurement.

RESULTS

Ectopic Expression of SHP-1, SHP-1C455S, and Bcl-2 Does Not Affect SSTR Function—We first established that overexpression of SHP-1, its inactive mutant, or Bcl-2 did not alter SSTR levels since the binding capacity determined by saturation binding analysis using [125I-Tyr3]OCT was similar in all cell lines and ranged from 356 ± 34 to 389 ± 65 fmol/mg protein. Likewise the affinity of [125I-Tyr3]OCT was also remained unaltered (Kd = 88–143 nM). OCT (100 nm) inhibited forskolin-stimulated cAMP by 47 ± 4% in both control and transfected MCF-7 cell lines (not shown).

Overexpression of SHP-1 Increases OCT-induced Apoptosis in MCF-7 Cells—To determine the effect of the overexpression of these proteins on SSTR-signaled apoptosis, we measured Annexin-V labeling in cells incubated in the absence or presence of 100 nm OCT for 24 h. Increased Annexin-V labeling, a characteristic of apoptotic cells, was seen in OCT-treated control cells and in cells expressing SHP-1 but not SHP-1C455S (Fig. 2A, compare panels B, D, and F). The ability of OCT to signal apoptosis was higher in SHP-1 transfected cells compared with control cells as seen from the −2.5-fold increase in Annexin-V labeling (66 ± 8 versus 27 ± 8%, respectively, mean ± S.E., n = 5). Expression of the vector alone in MCF-7 cells had no influence on the ability of OCT to signal apoptosis (not shown). OCT-induced cytotoxic signaling occurred earlier in SHP-1 transfected cells as evidenced by the formation of DNA fragments by 2 h compared to 6 h in untransfected cells; the extent of apoptosis at subsequent time points was higher in SHP-1 cells than in control cells (Fig. 2B).

OCT Induces Translocation of Cytosolic SHP-1 to the Membrane—In MCF-7 cells, SHP-1 was recruited to the membrane by OCT as revealed by immunoblot analysis (Fig. 3). Activities for hydrolizing pNPP in untreated and treated MCF-7 cells were 0.53 ± 0.04 versus 0.24 ± 0.02 nmol/mg of protein/min (cytosol) and 0.32 ± 0.03 versus 0.84 ± 0.06 nmol/mg of protein/...
min (membrane), respectively. Additionally we show that in SHP-1 cells, the enzyme protein was predominantly membrane-associated even in the absence of OCT treatment. Nevertheless, OCT induced a further increase in the membrane associated enzyme activity (1.69 ± 0.14 versus 1.38 ± 0.15 nmol/mg of protein/min, respectively, in the treated and untreated cells). By contrast, the translocation of SHP-1 to the membrane was blocked by the catalytically inactive mutant SHP-1C455S. In contrast to its effect on SHP-1, OCT did not alter the subcellular distribution of SHP-2 in MCF-7 cells (Fig. 4).

**OCT-signal, SHP-1-mediated Apoptosis in MCF-7 Cells Is Associated with Intracellular Acidification**—We have previously shown that activation of a cation-insensitive, acidic endonuclease occurs concomitantly with a decrease in pH in MCF-7 cells undergoing OCT-induced apoptosis (24, 25). Here we assessed the requirement of SHP-1 for OCT-induced intracellular acidification by measuring the pHi of cells. OCT-induced acidification as indicated by the increase in the fluorescence of the pH-sensitive dye carboxy-SNARF-1 at 580 nm relative to that at 640 nm was higher in SHP-1 expressing cells than in untransfected cells (Fig. 5, compare panels 2 and 4). By contrast, OCT failed to induce acidification in cells expressing SHP-1C455S (Fig. 5, panel 6). Intracellular pH was calculated from the ratios of fluorescence measured at 580 and 640 nm wavelengths (25, 44). Interestingly, the resting pHi was lower in the SHP-1 transfected cells and higher in the SHP-1C455S transfected cells (7.07 and 7.40, respectively) compared with that of 7.25 in MCF-7 cells (Fig. 5B). OCT-induced decrease in pHi in untransfected MCF-7 cells from 7.25 ± 0.07 to 7.2 ± 0.1 by 2 h and was maximal at 24 h (6.54 ± 0.06). By contrast, the rate and extent of OCT-induced acidification in SHP-1 overexpressing cells were higher: pHi decreased by 0.7 unit by 2 h and >1 unit after 24 h (pHi = 6.5 ± 0.2 and <6, respectively).

**Fas-mediated Cytotoxic Signalizing Requires SHP-1—Apoptosis induced following Fas ligation is associated with intracellular acidification in Jurkat cells (38, 45). Although it has been shown to induce apoptosis in some clones of MCF-7 cells (46–48), its effect on pHi in this cell line has not been reported. We therefore examined if Fas ligation induces acidification dependent apoptosis in MCF-7 cells used in this study. Fas-induced cell death could be detected by 5 h by the increase in the number of Annexin -V-positive cells (28 ± 3.5% compared with 3.7 ± 0.7% in the untreated cells, Fig. 6A). Fas mediated apoptosis correlated with cellular acidification (pHi 6.45 ± 0.07 compared with 7.25 ± 0.08 in untreated cells, Fig. 6B). Fas ligation failed to signal apoptosis when acidification was inhibited by pH clamping with nigericin. Additionally, we found that Fas-signalized acidification and apoptosis was SHP-1-dependent (Fig. 7, A and B). In cells overexpressing SHP-1, the number of apoptotic cells was ~2-fold higher compared with that seen in untransfected control cells (62.4 ± 8 versus 28.4 ± 4%). Fas ligation decreased the pHi of SHP-1 overexpressing cells to 6.3 ± 0.06 compared with 6.45 ± 0.07 in untransfected control cells. An increase in membrane-associated tyrosine phosphatase activity concomitant with a decrease in the cytosolic enzyme activity was seen in Fas-ligated cells (Fig. 8). Such a redistribution of the enzyme activity paralleled Fas-mediated induction of membrane translocation of SHP-1 (Fig. 8, inset).

**Bcl-2 Prevents D-[Trp8]SST-14-induced Intracellular Acidification and Apoptosis—**D-[Trp8]SST-14-induced apoptosis was detectable in untransfected and empty vector transfected cells (MCF-7 and MCF-VC), but not in cells transfected with Bcl-2 (MCF-7-Bcl-2) (Fig. 9A). Bcl-2 expression also prevented the formation of a hypodiploid cell population that could be discerned in MCF-7 and MCF-7-VC cells by flow cytometry following PI staining and by the presence of DNA laddering on agarose gels (data not shown). The average pHi was 7.25 ± 0.04 in both MCF-7 and MCF-7-VC cells (Fig. 9B). Interestingly, as
shown in this figure, MCF-7-Bcl-2 cells had a higher resting pH (7.45 ± 0.01). Following treatment with SST, the pH in cells expressing Bcl-2 was reduced by only 0.2 units, a value much less than the 0.7 unit drop in pH observed for MCF-7 and MCF-7-VC cells. The combination of these two effects resulted in a normalization of pH, but not acidification in MCF-7-Bcl-2 cells exposed to D-[Trp8]SST-14.

Bcl-2 Cannot Prevent Acidification-triggered Apoptosis—To investigate whether Bcl-2 can protect against apoptosis triggered by direct acidification, we examined the effect of clamping the cytoplasmic pH of MCF-7-Bcl-2 cells to 6.5 by incubation in acidic medium containing the proton ionophore nigericin. Under these conditions, acidification triggered apoptosis, as measured by DNA fragmentation, occurred in MCF-7-Bcl-2 cells to the same extent as was seen in MCF-7 cells (Fig. 10). Furthermore, this acidification-induced apoptosis is dependent on tyrosine phosphatase activity since the PTP inhibitor sodium orthovanadate prevented acidification triggered DNA fragmentation in both MCF-7 and MCF-7-Bcl-2 cells.

Our previous work suggests that NHE and H1-ATPase are involved in the pH changes that follow SST exposure, with NHE having the predominant effect (25). To determine if Bcl-2 influences the function of NHE we compared the effects of the NHE inhibitor EIPA on SST induced apoptosis in MCF-7-VC and MCF-7-Bcl-2 cells. EIPA lowered the pH in MCF-7-VC cells by 0.7 units; this decrease in pH was similar in magnitude to that seen with SST alone and the effect of both agents together was not significantly different than either alone in inducing intracellular acidification (Fig. 11A, lanes 2–4). Bcl-2 attenuated to a similar extent the intracellular acidification that resulted from exposure to either agent (Fig. 11A, compare lanes 5–7 with 1–3), but failed to inhibit the acidification in cells subjected to simultaneous treatment with D-[Trp8]SST-14 and EIPA (pH 6.65 ± 0.04, lane 8). As expected from their effects on acidification, apoptosis was seen to occur in MCF-7-VC cells treated with D-[Trp8]SST-14 or EIPA individually or together (Fig. 11B). By contrast, DNA fragmentation was detectable in MCF-Bcl-2 cells only when incubated with both D-[Trp8]SST-14 and EIPA.

Bcl-2 Does Not Prevent D-[Trp8]SST-14-induced Recruitment of SHP-1—A substantial recruitment of the normally cytosolic SHP-1 to the membrane with a concomitant increase in membrane-associated tyrosine phosphatase activity was seen in MCF-7 cells treated with D-[Trp8]SST-14 (Fig. 12, compare lanes 1 and 2 with lanes 5 and 6) consistent with our previous observations. In contrast to the effect of Bcl-2 on the pH changes associated with SST exposure, SST-induced membrane recruitment of SHP-1 and increased membrane associated phosphatase activity were unaffected by overexpression of Bcl-2 (Fig. 12, compare lanes 3 and 4 with lanes 7 and 8).
Bcl-2 Inhibits p53-mediated Induction of Bax—Another feature of SST-induced apoptosis in MCF-7 cells is SHP-1-dependent increase in p53 and Bax (21, 24, 25). By clamping MCF-7 to physiologic pH during exposure to SST, we have shown previously that the increased expression of these pro-apoptotic proteins is independent of intracellular acidification (21, 24, 25). These results suggest that expression of p53 and Bax represent a parallel pathway toward apoptosis rather than a consequence of SST-induced modifications in NHE activity. We therefore investigated the effect of Bcl-2 on the induction of p53 and Bax by D-[Trp8]SST-14. Exposure to SST increased the expression of wild type p53 equivalently in MCF-7, MCF-7-VC, and MCF-7-Bcl-2 cells (Fig. 13, top panel). However, a SST-induced increase in Bax expression was not seen in MCF-7-Bcl-2 cells (Fig. 13, bottom panel).

The present findings provide direct evidence demonstrating that SHP-1 is involved in SST- and Fas-mediated antiproliferative signaling that leads to intracellular acidification and apoptosis in MCF-7 cells. We showed that SHP-1 was recruited to the membrane upon OCT treatment in agreement with our previous reports (21, 25). The catalytically inactive mutant SHP-1C455S inhibited the action of OCT by blocking the translocation of SHP-1 to the membrane. These data reinforce the notion that association of SHP-1 with the membrane is a prerequisite for mediating the apoptotic signal of OCT (21, 25). When overexpressed, SHP-1 was constitutively membrane-associated and while its presence at the membrane did not initiate cytotoxic signaling by itself, it amplified the cytotoxic action of OCT. This effect is not peculiar to a particular cell line since we have observed similar effect of overexpressed SHP-1 on OCT-induced apoptosis and intracellular acidification in two other breast cancer cell lines (T47D and ZR-75–1, data not shown). Ligand-activated SSTCR can also negatively couple to the adenylyl cyclase-cAMP pathway to inhibit stimulated, but not basal, cAMP production (49). Here we found that alterations in SHP-1 levels did not alter the ability of OCT to inhibit forskolin-stimulated cAMP in MCF-7 cells. Moreover, the basal cAMP levels in the cells overexpressing SHP-1 or SHP-1C455S were comparable to that in untransfected MCF-7 cells. From these data we conclude that SHP-1-mediated antiproliferative signaling of SST is unlikely to be influenced by the other signaling pathways linked to the SSTR.

In the case of Fas signaling, the formation of a multiprotein complex called death-inducing signal complex consisting of oligomerized Fas, FADD, RIP, RAIDD, and sentrin has been shown to be necessary for apoptosis (50–55). Regulation of Fas-mediated apoptosis by tyrosine phosphatase has remained controversial. In the motheaten mice lacking SHP-1, Fas was found to be ineffective in signaling lymphocyte apoptosis (13, 14). Another protein-tyrosine phosphatase FAP-1 or PTP-1ε was reported to be involved in terminating Fas-mediated cytotoxic signaling (49). The involvement of both SHP-1 and FAP-1 in regulating Fas-signalced apoptosis has been questioned (15, 56). The present data demonstrating that Fas-mediated apoptosis is amplified by overexpression of SHP-1 and abrogated by its catalytically inactive mutant provide direct evidence that SHP-1 is necessary for the manifestation of apoptosis signaled by Fas ligation. Moreover, we showed that SHP-1 translocates...
of these agents decreased the pH (mean \( \pm \) S.E., \( n = 6 \)).

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The mechanism underlying the membrane translocation of SHP-1 induced by SSTR activation or Fas ligation remains to be elucidated. The membrane translocation of SHP-1 may be modulated by the binding of the enzyme to specific tyrosine-phosphorylated proteins. It is yet to be clarified as to whether the active or inactive enzyme associates with the cell membrane. The fact that the catalytically inactive mutant blocked the OCT-induced recruitment of SHP-1 to the membrane indicates that the catalytic activity of SHP-1 may be necessary for its localization at the membrane. However, other studies suggest that its redistribution to the membrane may occur without prior activation, probably involving its binding through SH2 domains to membrane protein(s) or to membrane phospholipids (5, 57, 58). The membrane-associated phosphatase may then be stimulated by anionic phospholipids as was observed in vitro (57). The importance and contribution of the NH2-terminal SH2 domains as well as residues within the catalytic domain of SHP-1 for its ability to translocate to the membrane are currently under investigation.

A number of proteins that are acted upon by SHP-1 have been identified: these include proteins recruited through Shc and Grb2 in T lymphocytes and several immunoreceptor tyrosine-based inhibitory motif containing proteins including CD22, CD72, paired immunoglobulin-like receptor B, and Killer cell inhibitory receptor (59–70). By interacting with these proteins SHP-1 attenuates their ability to trigger apo-
pesis. However, to date, putative pro-apoptotic molecule(s) that is/are activated by SHP-1 through dephosphorylation have not been identified. From our results, we assume that such molecule(s) may exist and become transducer(s) of apoptotic signal of SST upon dephosphorylation by membrane-associated SHP-1.

The cytotoxic signals initiated via SSTR and Fas require SHP-1-dependent inhibition of pH homeostasis to permit the execution of downstream apoptotic events. We showed that the extent of acidification signaled through SSTR and Fas was higher in SHP-1-transfected cells. Additionally, the constitutive membrane association of overexpressed SHP-1 triggered a decrease in the pH_i of the resting, untreated cells. By contrast, dominant negative mutant SHP-1C455S which did not associate with the membrane prevented its association of SHP-1 with the membrane, caused the increase of the resting pH_i in cells, and abrogated the pH lowering effect signaled by SSTR activation or Fas ligation. We have recently shown that SHP-1-mediated induction of p53 by SST is not acidification-dependent or Fas ligation. We have recently shown that SHP-1-dependent modulation of these proton extrusion processes. Taken together, these data suggest that SHP-1 may regulate multiple steps in apoptotic signaling, namely the induction of pro-apoptotic molecules that precede decrease in pH_i, pH homeostasis, and apoptotic events that occur following acidification.

SSTR-mediated, SHP-1-dependent cytotoxic signaling is inhibited by Bcl-2 prior to, but not, following acidification. We have previously shown that the acidification and apoptosis that follows EIPA exposure is also inhibited by a dominant negative mutant of SHP-1 (25). EIPA and SST are each effective at following EIPA exposure is also inhibited by a dominant negative mutant of SHP-1 (25). EIPA and SST are each effective at acidification. These data combined with our previously reported findings (24, 25, 43, 44) show that SHP-1 is required for regulating multiple apoptotic events both before and after acidification whereas Bcl-2 inhibits SHP-1-dependent apoptosis solely by preventing the decrease in pH_i.

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In summary, we have shown that cytotoxic signaling induced through SSTR activation as well as by Fas ligation is mediated by membrane-associated SHP-1. The mere presence of SHP-1 at the membrane does not, by itself, trigger acidification and apoptosis but amplifies the apoptotic response to Fas ligation and SSTR activation. These data suggest that molecules recruited by the SSTR and Fas may function as putative substrates of SHP-1 that upon dephosphorylation promote cellular acidification thereby facilitating apoptotic signaling. Bcl-2 prevents cytotoxic signaling of SST downstream of recruitment of SHP-1 to the membrane and induction of p53 induction to inhibit p53-mediated transactivation of Bax expression and, to attenuate acidification. Its ability to inhibit acidification is due both to a novel effect of Bcl-2 of increasing the resting pH_i and to attenuation of SHP-1-mediated decrease in pH_i in MCF-7 cells. It cannot, however, prevent apoptotic events that follow acidification. These data combined with our previously reported findings (24, 25, 43, 44) show that SHP-1 is required for regulating multiple apoptotic events both before and after acidification whereas Bcl-2 inhibits SHP-1-dependent apoptosis solely by preventing the decrease in pH_i.
