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Interdependent Regulation of Intracellular Acidification and SHP-1 in Apoptosis

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ABSTRACT
The G protein-coupled receptor agonist somatostatin (SST)-induces apoptosis in MCF-7 human breast cancer cells. This is associated with induction of wild-type p53, Bax, and an acidic endonuclease. We have shown recently that its cytotoxic signaling is mediated via membrane-associated SHP-1 and is dependent on decrease in intracellular pH (pH<sub>i</sub>) to 6.5. Here we investigated the relationship between intracellular acidification and SHP-1 in cytotoxic signaling. Clamping of pH<sub>i</sub> at 7.25 by the proton-ionophore nigericin abolished SST-signaled apoptosis without affecting its ability to regulate SHP-1, p53, and Bax. Apoptosis could be induced by nigericin clamping of pH<sub>i</sub> to 6.5. Such acidification-induced apoptosis was not observed at pH<sub>i</sub> < 6.0 or > 6.7. pH<sub>i</sub>-dependent apoptosis was associated with the translocation of SHP-1 to the membrane, enhanced in cells overexpressing SHP-1, and was abolished by its inactive mutant SHP-1C455S. Acidification caused by inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger and H<sup>+</sup>-ATPase (pH<sub>i</sub> = 6.55 and 6.65, respectively) also triggered apoptosis. The effect of concurrent inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger and H<sup>+</sup>-ATPase on pH<sub>i</sub> and apoptosis was comparable with that of SST. Acidification-induced, SHP-1-dependent apoptosis occurred in breast cancer cell lines in which SST was cytotoxic (MCF-7 and T47D) or not (MDA-MB-231). We conclude that: (a) SST-induced SHP-1-dependent acidification occurs subsequent to or independent of the induction of p53 and Bax; (b) SST-induced intracellular acidification may arise due to inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger and H<sup>+</sup>-ATPase; and (c) SHP-1 is necessary not only for agonist-induced acidification but also for the execution of acidification-dependent apoptosis. We suggest that combined targeting of SHP-1 and intracellular acidification may lead to a novel strategy of anticancer therapy bypassing the need for receptor-mediated signaling.

INTRODUCTION
The nontransmembrane tyrosine phosphatase SHP-1 is implicated in the signaling of apoptosis in lymphoid cells and in breast cancer cells (1–4). We have shown that the recruitment of cytosolic SHP-1 to the membrane is an early event in the antiproliferative signaling of the G protein-coupled receptor agonist SST<sub>2</sub> in MCF-7 breast cancer cells (4). The SHP-1-dependent growth-inhibitory action of SST leads to induction of wild-type p53, Bax, intracellular acidification, and apoptosis in MCF-7 cells (2–4). SST-induced acidification could be inhibited by the catalytically inactive mutant of SHP-1, suggesting that SHP-1 may modulate pH homeostasis (2). The specific pH regulatory event(s) that may be inhibited in an SHP-1-dependent manner remains unknown. Likewise, it is not known whether intracellular acidification affects the subcellular distribution and activity of SHP-1. A decrease in pH<sub>i</sub> could arise as a consequence of disrupted regulation of proton extrusion pathways that include BAF-1-sensitive H<sup>+</sup>-ATPase and amiloride-inhibitable Na<sup>+</sup>/H<sup>+</sup> antiporter (NHE), which are maximally active at pH<sub>i</sub> < 6.7, and a Zn<sup>2+</sup>-inhibitable H<sup>+</sup> conductance that is insensitive to BAF-1 and amiloride (5–9). Additional pH regulatory mechanisms involving sodium-dependent Cl<sup>−</sup>/HCO<sub>3</sub> exchange that functions optimally at pH<sub>i</sub> ~ 6.9 have also been described (10). Although modulation of pH-regulatory pathways by SHP-1 has not been shown, there is evidence that NHE and H<sup>+</sup>-ATPase could be activated in the presence of phosphotyrosine phosphatase inhibitors (11–14). To define the relationship between SHP-1 and pH<sub>i</sub>, we investigated: (a) the requirement of a change in pH<sub>i</sub> for SST-induced translocation of SHP-1 to the membrane in MCF-7 cells; (b) the effect of (direct) acidification on cellular distribution of SHP-1 and apoptosis; (c) the ability of selective inhibitors of distinct proton extrusion pathways to induce intracellular acidification and apoptosis; and (d) the effect of acidification in two other breast cancer cell lines that displayed cytotoxic response to SST (T47D) or not (MDA-MB-231). We report here that SST-induced intracellular acidification occurs distal to or independent of changes in pH<sub>i</sub>, p53, and Bax and may arise due to inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger and H<sup>+</sup>-ATPase. Additionally, SHP-1 is necessary not only for agonist-induced acidification but also for the execution of acidification-dependent apoptosis.

MATERIALS AND METHODS
The human breast cancer cell lines MCF-7 (clone HTB22), T47D, and MDA-MB-231 were obtained from American Type Culture Collection (Bethesda, MD). The somatostatin analogue [D-Trp<sub>8</sub>]SST-14 was purchased from Bachem (Torrance, CA). pNPP and DIDS were obtained from Sigma Chemical Co. (St. Louis, MO). Annexin V apoptosis detection kit was purchased from Boehringer Mannheim Canada (Montreal, Quebec). EIPA and BAF-1 were supplied by Research Biochemicals International (Natick, MA) and ICN (Costa Mesa, CA), respectively. All other chemicals used were of analytical grade and were obtained from regular commercial sources.

Cell Culture. MCF-7 cells were plated in 75-cm<sup>2</sup> culture flasks and grown in MEM containing nonessential amino acids and supplemented with 10% FBS and 10 mg/ml bovine insulin. MCF-7 cells stably expressing SHP-1 or its catalytically inactive mutant SHP-1C455S were established as described previously and maintained in the above medium containing 400 µg/ml G-418 (2). T47D cells were maintained in RPMI 1640 supplemented with 10% FBS, and MDA-MB-231 cells were cultured in Liebovitz L-15 medium containing 10% FBS. Inhibition of individual proton extrusion pathways in MCF-7 cells was monitored using 2.5 μM Zn<sup>2+</sup> (inhibitor of BAF-1 and amiloride-insensitive proton transport), 250 μM DIDS (inhibitor of Cl<sup>−</sup>/HCO<sub>3</sub><sup>−</sup> channel), 100 nM BAF-1 (inhibitor of H<sup>+</sup>-ATPase), and 100 nM EIPA (inhibitor of NHE). When investigating the effect of intracellular acidification, the cells were cultured in the presence of the proton ionophore nigericin (10 µM) in medium buffered to the required pH ranging from 7.25 to 5.0. Nigericin-containing media were supplemented with 140 mM K<sup>+</sup>, which facilitates pH equilibration (15, 16).

Measurement of PTP Activity SHP-1 Immunoblot Analysis. To assess the effect of SST on translocation of SHP-1, flasks containing an equal number (5 × 10<sup>6</sup>) of cells were incubated for 1 h in the presence or absence of 100 nM [D-Trp<sub>8</sub>]SST-14. Cells were washed in PBS and resuspended in buffer containing 200 mM mannitol, 68 mM sucrose, 50 mM HEPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 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 × g.
Measurement of Intracellular pH. Cells were incubated at 37°C in the absence or presence of 100 nM [D-Trp8]SST-14 for 24 h and loaded with 10 μM acetoxyethyl ester derivative of SNARF-1 during the final hour (2, 17). The cells were then scraped, washed, and maintained at 37°C in a Becton Dickinson FACStar Vantage cytometer. Intracellular carboxy SNARF-1 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 ratios of the samples to a calibration curve of intracellular pH generated by incubation of carboxy-SNARF-1 loaded cells in nigericin containing buffers with adjusted pH ranging from 8.0 to 6.25 (2). Cells with fluorescence of <50 units were excluded in the calculation of the ratio of the emissions at 580 and 640 nm.

RESULTS

[D-Trp8]SST-14 induced a decrease of 0.8 unit in intracellular pH of MCF-7 cells (pHi = 6.42 ± 0.06 compared with 7.25 ± 0.07 in control cells) and apoptosis (Fig. 1). Addition of the proton ionophore nigericin to the incubation medium abolished the pH lowering effect of [D-Trp8]SST-14 (Fig. 1A) as well as its ability to induce apoptosis (Fig. 1B and C). Prevention of intracellular acidification by nigericin did not affect [D-Trp8]SST-14-induced increase in membrane-associated tyrosine phosphatase activity or the translocation of SHP-1 to the membrane (Fig. 2). Likewise, the inductive effect of [D-Trp8]SST-14 on wild-type p53 and Bax was also not dependent on pHi (Fig. 3). Prevention of intracellular acidification by nigericin did not affect [D-Trp8]SST-14-induced increase in membrane-associated tyrosine phosphatase activity by [D-Trp8]SST-14 (means; bar, SE; n = 6). Insets, immunoblot demonstrating that recruitment of SHP-1 to the membrane by [D-Trp8]SST-14 was not affected by prevention of acidification (representative of three experiments).
To determine whether acidification per se could induce apoptosis, we clamped the pH by incubating cells in nigericin-containing media buffered from 7.0 and 5.0. There was a rapid equilibration of intracellular and extracellular pH under these conditions (details not shown). We analyzed the effect of acidic pH after 4 h because there was increasing detachment of cells at longer time periods. DNA fragmentation was observed only in cells with a pHi of 6.5 and, to a lesser extent, in cells with pHi of 6.0 (Fig. 4A). No apoptosis was evident in cells with a pHi >7.0 or in cells with pHi of <6.0. To our surprise, acidification-induced apoptosis in MCF-7 cells was abolished by the tyrosine phosphatase inhibitor orthovanadate (Fig. 4B). We therefore assessed the phosphatase activity and the distribution of SHP-1 in the cytosolic and membrane fractions as a function of pHi. An increase in membrane-associated tyrosine phosphatase activity was seen in cells with pHi of 6.5 and 6.0 (Fig. 5A). A pH-dependent redistribution of SHP-1 was observed in cells with pHi of 6.5 and 6.0 but not in cells with more acidic or alkaline pHi (Fig. 5B). To confirm the SHP-1 dependency of acidification-induced cytotoxic signaling, we compared the effect of lowering the pHi to 6.5 in cells overexpressing the wild-type enzyme or its catalytically inactive mutant, SHP-1C455S. As shown in Fig. 6, acidification-induced DNA fragmentation was enhanced by the overexpression of SHP-1 and was completely abolished by SHP-1C455S.

To determine which of the proton extrusion pathways is necessary for acidification, we compared the effect of blockers of specific pH regulatory mechanisms with that of [D-Trp8]SST-14 (Fig. 7). The cellular pH did not decrease in response to Zn2+. The Cl-/HCO3− channel inhibitor DIDS caused mild acidification (pHi = 6.87 ± 0.07 compared with 7.24 ± 0.06 in the control; Fig. 7A). By contrast, the H+-ATPase inhibitor BAF-1 and the NHE inhibitor EIPA lowered the intracellular pH by 0.60 and 0.69 units (pHi = 6.65 ± 0.06 and 6.55 ± 0.053, respectively), BAF-1 and EIPA together decreased the pHi to almost the same level as did [D-Trp8]SST-14 (pHi = 6.48 ± 0.04 and 6.42 ± 0.05, respectively). Apoptosis was seen to occur in cells treated with BAF-1 and EIPA but not with Zn2+ and DIDS (Fig. 7B). DNA fragmentation was greater in cells treated with EIPA than with BAF-1. The degree of apoptosis in cells treated simultaneously with BAF-1 and EIPA was comparable with that induced by [D-Trp8]SST-14. To determine which of these channels are modulated by SHP-1, we compared the effect of DIDS, BAF-1, and EIPA in MCF-7 cells overexpressing the wild-type SHP-1 or its catalytically inactive mutant. The ability of DIDS to decrease the pHi was not affected by the overexpression of either SHP-1 or SHP-1C455S (Fig. 8). By contrast, BAF-1 and EIPA, which were fully modulated by SHP-1, we compared the effect of DIDS, BAF-1, and EIPA in MCF-7 cells overexpressing the wild-type SHP-1 or its catalytically inactive mutant. The ability of DIDS to decrease the pHi was not affected by the overexpression of either SHP-1 or SHP-1C455S (Fig. 8). By contrast, BAF-1 and EIPA, which were fully modulated by SHP-1, we compared the effect of DIDS, BAF-1, and EIPA in MCF-7 cells overexpressing the wild-type SHP-1 or its catalytically inactive mutant. 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capable of triggering intracellular acidification in cells overexpressing SHP-1, were ineffective in acidifying SHP-1C455S-expressing cells.

Finally, we examined the ability of [D-Trp^8]SST-14 to induce intracellular acidification and apoptosis in two other breast cancer cell lines (T47D and MDA-MB-231). [D-Trp^8]SST-14 induced apoptosis in T47D but not MDA-MB-231 cells (Fig. 9A). By contrast, both cells underwent apoptosis when incubated in media buffered to pH 6.5 in the presence of nigericin. Furthermore, acidification-induced apoptosis was inhibited by orthovanadate in both cell types. Additionally, [D-Trp^8]SST-14 induced translocation of SHP-1 to the membrane in T47D but not in MDA-MB 231 cells, whereas there was an increase in membrane-associated SHP-1 in both cells in which the pH_i was clamped to 6.5 (Fig. 9, B and C). [D-Trp^8]SST-14-induced apoptosis in T47D cells was associated with intracellular acidification (pH_i = 6.46 ± 0.06) comparable with that seen in MCF-7 cells. Interestingly, despite its inability to signal apoptosis in MDA-MB-231...
incubated in the absence or presence of 100 nM peptide for 24 h.

This is, to our knowledge, the first tendency between SHP-1 activation and acidification occurred over a narrow range of pHi (6.7–6.0). Such a pH-dependent regulation of SHP-1 to the membrane restored the pH homeostasis (2, 4). The inability of DIDS to trigger apoptosis, although it decreased the pH to 6.9, suggests that inhibition of Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\)^\(^-\) channel is insufficient to trigger the apoptotic process. [D-Trp\(^8\)]SST-14 decreased the pH of MDA-MB-231 cells to 6.95, the same extent as did DIDS. The modest decrease in pH induced by DIDS was comparable in MCF-7 cells expressing the wild-type enzyme or the inactive mutant (data not shown), suggesting that SST may inhibit Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\)^\(^-\) channel in an SHP-1-independent manner.

NHE and H\(^+\)-ATPase are present in tumor cells, and their inhibition leads to intracellular acidification and apoptosis (18–22). The present data showing that BAF-1 and EIPA trigger acidification and apoptosis in MCF-7 cells is in agreement with these findings. We showed that acidosis resulting from the inhibition of NHE by EIPA was greater than that due to inhibition of H\(^+\)-ATPase by BAF-1. The degree of acidification induced by EIPA was greater than that caused by BAF-1, suggesting that SST may exert a greater inhibitory effect on NHE compared with H\(^+\)-ATPase. Alternatively, this may reflect the possibility that NHE is more active in pH regulation compared with H\(^+\)-ATPase. The extent of acidification and apoptosis induced by these inhibitors together was comparable with that seen in [D-Trp\(^8\)]SST-14-treated cells. Thus, inhibition of both H\(^+\)-extrusion pathways may be required for the distal events of SST-induced cytotoxic signaling. The present finding that SHP-1C455S prevented the acidification caused by EIPA and BAF-1 suggests that the activity of both NHE and H\(^+\)-ATPase is modulated by SHP-1. Interestingly, the resting pH of MCF-7 cells was decreased by overexpressed SHP-1, whereas it was increased by SHP-1C455S (7.06 ± 0.08 and 7.37 ± 0.06, respectively, compared with the value of 7.25 ± 0.07 in untransfected cells). These data, to our knowledge, provide the first documentation of SHP-1-dependent regulation of pH homeostasis through NHE and H\(^+\)-ATPase. Although the specific involvement of SHP-1 in intracellular acidification has not been described previously, PTP inhibitors have been shown to induce multiple pH regulatory pathways (H\(^+\)-ATPase, NHE, and a Zn\(^++\)-sensitive H\(^+\) conductance; Ref. 11). Vanadate was also capable of activating NHE in a variety of systems (12–14). The activity of NHE and H\(^+\)-ATPase has been shown to be modulated by serine/threonine, but not tyrosine, phosphorylation (23). BAF-1 and EIPA failed to trigger acidification in cells expressing SHP-1C455S to the same extent as they did in MCF-7 cells (data not shown). We do not know at the present time how SST inhibits the activity of these proteins in an SHP-1-dependent manner. It remains to be established as to whether SHP-1 regulates the activity of both NHE and H\(^+\)-ATPase directly or indirectly through other proteins. Six isoforms of NHE have been identified (23). NHE-1 and NHE-6 are ubiquitously expressed in most cell types. NHE-1 is found on the plasma membranes of virtually all cells and is believed to control cytosolic pH and cell volume regulation. NHE-2, NHE-3, NHE-4, and NHE-5 are more restricted in their expression patterns, reflecting specialized functions, and display differences in their apparent H\(^+\)-sensitivity and relative, but not absolute, selectivity toward different inhibitors (23, 24). Such differences may arise due to a possible intracellular rather than plasma membrane localization of some NHEs. Indeed, NHE-3 has been shown to be predominantly juxtanuclear or endosomal (25). The expression patterns and subcellular localization of the NHE isoforms in MCF-7 cells remain to be determined; hence, we do not know at the present time which specific NHE isoform(s) is inhibited by SST.

Cytotoxic signaling by SST is dependent not only on the recruitment of SHP-1 to the membrane but also on intracellular acidification. Such an effect seen in breast cancer cells is signaled in a subtype-selective manner uniquely via hSSTR subtype 3 in heterologous expression system in CHO-K1 cells, whereas the other four hSSTR subtypes elicit cytotoxic response, resulting in G1 cell cycle arrest but...
not apoptosis (26, 27). The presence of hSSTR3 has been detected by reverse transcription-PCR in many, but not all, cancers of the breast and other tissues (28–30). In tumors that do not express this subtype, SST analogues will fail to trigger apoptosis and may, therefore, have limited therapeutic potential. We have shown that intracellular acidification is sufficient to trigger apoptosis in an SHP-1-dependent manner, even in cells that do not undergo apoptosis in response to SST. The pH of tumor cells is generally higher than that of the extracellular environment (31). Indeed, compounds that reverse the imbalance between the intra- and extracellular pH of tumor cells in vivo have been developed as a potential new class of anticancer drugs (32, 33). Specifically, inhibitors of proton extrusion have been shown to inhibit tumor cell growth (34–38). The importance of tyrosine phosphatases in antiapoptotic signaling has been recognized but has not yet been exploited for their therapeutic potential. Our finding that SHP-1 is involved in both receptor-mediated and acidification–induced apoptotic signaling underscores its potential usefulness in cancer therapy.

In summary, we have shown that SST-induced acidification appears to be due to SHP-1-dependent inhibition of NHE and H⁺-ATPase and SHP-1-dependent inhibition of Na⁺-dependent Cl⁻/HCO₃⁻ channel. SST-induced acidification occurs independent of or distal to the induction of wild-type p53 and Bax. SHP-1 is necessary not only for agonist-induced acidification but also for apoptosis triggered by a decrease in pH. A pH-dependent membrane association and activation of SHP-1 occurs in breast cancer cell lines that undergo apoptosis in response to SST (MCF-7 and T47D) or not (MDA-MB 231). These findings reveal a hitherto unrecognized interdependency between intracellular acidification and SHP-1-dependent signaling of apoptosis. We suggest that combined targeting of SHP-1 and intracellular acidification may lead to a novel strategy of anticancer therapy, bypassing the need for receptor-mediated signaling.

ACKNOWLEDGMENTS

We thank S. Schiller and K. MacDonald for assistance with flow cytometry.

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