Intracellular Signaling Mechanisms Leading to Synergistic Effects of Endothelin-1 and Stem Cell Factor on Proliferation of Cultured Human Melanocytes

CROSS-TALK VIA TRANS-ACTIVATION OF THE TYROSINE KINASE c-KIT RECEPTOR

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We previously reported that activation of mitogen-activated protein kinase (MAPK) is involved in the mitogenic stimulation of normal human melanocytes (NHMC) by endothelin-1 (ET-1). In the present study, we determined signaling mechanisms upstream of MAPK activation that are involved in ET-1 stimulation and their synergism with stem cell factor (SCF). Pretreatment of cultured NHMC with ET receptor antagonists, pertussis toxin, a specific phospholipase C inhibitor (U73122), or a protein kinase C inhibitor (calphostine) blocked a transient tyrosine phosphorylation of MAPK induced by ET-1, whereas the addition of a calcium chelator (BAPTA) failed to inhibit tyrosine phosphorylation of MAPK. Treatment with ET-1 and SCF together synergistically increased DNA synthesis, which was accompanied by synergism for MAPK phosphorylation. The time course of inositol 1,4,5-trisphosphate formation revealed that there is no difference in the level of inositol 1,4,5-trisphosphate stimulated by ET-1 + SCF or by ET-1 alone. Evaluations of the serine phosphorylation of MEK and Raf-1 activity showed a synergistic effect in SCF + ET-1-treated NHMC. Stimulation with SCF + ET-1 induced a more rapid and stronger tyrosyl phosphorylation of proteins corresponding to p52 and p66 Shc than did stimulation with SCF only, and this was accompanied by a stronger association of tyrosine-phosphorylated Shc with Grb2. Interestingly, a more rapid and marked tyrosine phosphorylation of c-kit was also detected in NHMC-treated with SCF + ET-1 than NHMC treated with SCF only. These data indicate that the synergistic cross-talk between ET-1 and SCF-1 signaling is initiated through the pathway of tyrosine phosphorylation of c-kit, which results in the enhanced formation of the Shc-Grb2 complex which leads in turn to the synergistic activation of the Ras/Raf-1/MEK/MAP kinase loop.

In the long course of studying paracrine mechanisms involved in epidermal hyperpigmentary disorders, we have found that endothelin-1 (ET-1) plays a central role in UVB-induced pigmentation (1, 2) and in the accentuated pigmentation of senile freckles. Furthermore, stem cell factor (SCF) plays an important role in the increased pigmentation of the epidermis overlying benign fibroblastic tumors in dermatofibromas as well as in UVB-induced pigmentation. We have also observed that aged fibroblasts in culture produce a larger amount of SCF than do younger ones (3), which probably provides a basis for the tendency of aged skins to be more sensitive to environmental stimuli and to be easily induced to epidermal hyperpigmentation. SCF is also known as a stimulator for epidermal hyperpigmentation in mastocytosis where mast cells undergo hyperproliferation in response to the soluble type of SCF derived from keratinocytes (4).

In inherited pigmentary diseases such as piebaldism and Hirschsprung disease, mutations of c-kit (5–7) or endothelin B receptor (8, 9), respectively, have been documented. In Waardenburg syndrome type 2, mutation of microphthalmia-associated transcription factor is considered to be a central event leading to the dysfunction or loss of melanocytes (10, 11). Recently, the c-kit signaling pathway was found to be upstream of microphthalmia-associated transcription factor transcription through phosphorylation by MAP kinase (MAPK) (12). In mast cells, many reports (13, 14) have described a strong link between c-kit expression and microphthalmia-associated transcription factor transcriptional function. Furthermore, a disease termed Shah-Waardenburg syndrome, which combines the Waardenburg type 2 and Hirschsprung phenotypes, shows defects in microphthalmia-associated transcription factor and in the endothelin B receptor (15).

Thus, it is likely that signaling pathways stimulated by binding of ET-1 and SCF to their corresponding receptors have at least some common pathways and are working in a coordinated fashion to regulate melanocyte function. In relation to this, we have recently found that the proliferation of cultured human melanocytes (NHMC) induced by ET-1 is synergistic-
cally enhanced by the concomitant addition of SCF (16).

Endothelins are unique mitogens and melanogens for human melanocytes (1, 2, 17). These cellular actions are known to be initiated by binding of ET-1 to G-protein-coupled ET<sub>R</sub> receptor, followed by sequential signaling processes consisting mainly of protein kinase C (PKC) and MAPK (16, 17). SCF stimulation is important for the survival and proliferation of several cell types in the hematopoietic system (18, 19), where it may function in combination with other growth factors. In human melanocytes, SCF alone is sufficient to sustain proliferation under serum-free conditions (16). SCF binding to the c-kit receptor mediates dimerization, activation of its intrinsic tyrosine kinase activity, and autophosphorylation (20). The activated receptor then phosphorylates various substrates and associates with various signaling molecules, including phosphatidylinositol 3'-kinase (PI 3-kinase), the Shc and Grb2 adapter proteins, and the guanine nucleotide exchange factor, SOS, all of which lead to the activation of the Ras-MAPK pathway (21–23). In the epidermis, the most abundant cells which surround melanocytes are keratinocytes, that are known to produce increased amounts of SCF and ET-1 in response to several stimuli (24). Therefore, it is of particular value to clarify the cross-talk mechanism between ET-1 and SCF-mediated signaling in human melanocytes because each of those specific signaling pathways has been associated with the physiological stimulation of melanogenesis (16). The aim of our present study was to evaluate the mechanisms by which ET signaling activates MAPK and its underlying synergism with SCF. We now report that the synergistic cross-talk between SCF and ET-1 signaling is initiated through the pathway of trans-activation of c-kit, including its enhanced tyrosine phosphorylation, during the SCF-induced activation process. This results in an increase in the formation of the Shc-Grb2 complex, which leads in turn to synergistic activation of the Ras/Raf-1/MEK/MAP kinase loop.

**EXPERIMENTAL PROCEDURES**

**Materials—**NHMC were obtained from Morinaga Co. Endothelin derivatives were purchased from Sigma. Anti-phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnology Inc. (New York), αERK1/2, α-phospho-specific ERK1/2, αMEK, α-phospho-specific antibodies from New England Labs Inc., α-c-kit and αGrb2 antibodies were from Santa Cruz Biotech, Inc., and α-Shc antibodies and anti-phosphotyrosine (clone RC20) from Transduction Lab. All other chemicals were of reagent grade.

**Cell Culture—**NHMC were maintained in modified MCDB 153 growth medium supplemented with 1 ng/ml recombinant basic fibroblast growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate, antibiotics (50 μg/ml gentamycin and 0.25 μg/ml amphotericin B), 0.5% fetal calf serum, and 0.2% bovine pituitary extract at 37 °C under a 5% CO<sub>2</sub> atmosphere. In experiments to evaluate cellular effects or signaling changes, NHMC were seeded in culture trays at a density of 5–8 × 10<sup>4</sup> cells/ml, and were cultured in keratinocyte-SFM (Life Technologies, Inc.) containing bovine pituitary blast growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate, antibiotics (50 μg/ml gentamycin and 0.25 μg/ml amphotericin B), 0.5% fetal calf serum, and 0.2% bovine pituitary extract at 37 °C under a 5% CO<sub>2</sub> atmosphere. In experiments to evaluate cellular effects or signaling changes, NHMC were seeded in culture trays at a density of 5–8 × 10<sup>4</sup> cells/ml, and were cultured in keratinocyte-SFM (Life Technologies, Inc.) containing bovine pituitary extract for 48 h, then treated with reagents at various concentrations.

**DNA Synthesis—**NHMC cultured in 96-well trays were incubated with ET-1 and/or SCF at concentrations of 0 to 100 nm. Twenty hours later, the cells were labeled for 4 h with 1.0 μCi/ml [3H]thymidine. After three washes with phosphate-buffered saline, the cells were trypsinized and harvested on a glass fiber filter, washed three times with distilled water, and twice with ice-cold ethanol, then dried. The radioactivity on the filter was directly measured using MATRIX 96 (Packard Biotechnology Co.).

**Measurement of Inositol 1,4,5-Trisphosphate (IP<sub>3</sub>)—**These techniques were performed as reported previously (2). Briefly, for IP<sub>3</sub> assay, cells were seeded in 24-well culture trays at a density of 3 × 10<sup>5</sup> cells/ml and were incubated for 24–48 h. The cells were then treated with 0.5 μg/ml of 4-aminopyridine and the MCDB 153 medium containing 10 mM LiCl was added and incubated for 10 min at 37 °C before stimulation. The ligand stimulation was terminated at designated times by adding 10% perchloric acid and the samples were kept on ice for 15 min. After neutralization with ice-cold 1 M KOH for 60 min on ice, the samples were centrifuged at 2,000 × g for 10 min to remove KCIO, precipitate. The supernatants (100 μl each) were subjected to IP<sub>3</sub> assay using the IP<sub>3</sub> assay kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) (2). The content of IP<sub>3</sub> in each sample was quantitatively determined from a calibration curve established using the binding protein specific for IP<sub>3</sub> and [3H]IP<sub>3</sub>. Western Immunoblotting—These techniques were performed as reported previously (25, 26). Lysates (20 μg/lane) or immunoprecipitated complex were separated by 10% SDS-PAGE and then transferred to Immobilon-P PVDF membranes (Millipore, Schisbron, Germany). Membranes were blocked with 3% bovine serum albumin in Tris-buffered saline containing Tween 20 (TBS-T) buffer (20 mM Tris-HCl, pH 7.2, 0.14 μM NaCl, 0.1% Tween 20) for 3 h at room temperature, and then probed with primary antibodies in TBS-T buffer. After washing, blots were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) for 1 h, and signals were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech).

**Immunoprecipitation—**These techniques were performed as reported previously (27, 28). Grb2, Shc, and c-kit were immunoprecipitated from whole cell lysates by incubation with 4 μg of antibodies for 2 h at 4 °C. The resultant immune complexes were then precipitated with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. The pellets were washed three times with RIPA buffer, once with phosphate-buffered saline, resuspended in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM dithiothreitol, 0.1% (w/v) bromophenol blue), and heated at 95 °C for 5 min. Immunoprecipitated and associated proteins were detected with immunoblotting as described above.

**Assay of Raf-1—**Raf-1 was assayed according to the method reported previously (29) by its ability to activate MAPK kinase, which was then assayed by the activation of MAPK (29). An aliquot of each cell lysate was added to 5 μl of protein G-Sepharose conjugated to 1.0 μg of Raf-1 antibody (Transduction Laboratories) and was incubated for 60 min at 4 °C on a shaking platform. The suspensions were centrifuged for 1 min at 14,000 × g, the supernatants discarded, and the immunoprecipitates washed twice with 1.0 ml of buffer (20 mM Tris acid, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonl fluoride, and leupeptin 5 μg/ml), twice with 1.0 ml of buffer (50 mM Tris-HCl, pH 7.5, 0.03% Brij 35, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol) and then assayed as described below. Eighteen μl of buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 25 mM sodium β-glycerophosphate, 5 mM PCK inhibitor peptide (Upstate Biotechnology Inc.), 0.5 mM PKA inhibitor peptide (Upstate Biotechnology Inc.), 5 mM compound R24571 (Upstate Biotechnology Inc.), 20 mM magnesium chloride, and 0.13 mM ATP) containing 0.2 μg of glutathione S-transferase-MAPKK1 and 0.7 μg of glutathione S-transferase-MAPK was added to immunoprecipitates by Raf-1 antibody and, after incubation for 30 min at 30 °C on a shaking platform, 2 μl aliquot was added to 15 μl of 20 mM MOPS (pH 7.2), 5 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 25 mM sodium β-glycerophosphate, MBP (0.67 mg/ml), 25 mM magnesium chloride, and 0.1 mM [γ-<sup>32</sup>P]ATP (∼1000 Ci/mmol). After incubation for 10 min at 30 °C, the incorporation of phosphate into MBP was determined by autoradiography of MBP separated with SDS-PAGE.

**RESULTS**

**Synergistic Stimulatory Effects of the Combination of SCF and ET-1 on DNA Synthesis—**When ET-1 and SCF are concomitantly added at constant and varied concentrations, respectively, there is a synergistic stimulation of DNA synthesis in NHMC (Fig. 1A). At ET-1 and SCF concentrations of 10 nm each, a marked synergistic stimulation of DNA synthesis is elicited with a 62-fold increase relative to the 1.2- and 7.2-fold increases elicited by SCF or ET-1 treatments alone, respectively.

**ET Binding Initiated Signaling Pathway Leading to the Ac-
In order to clarify whether the activation of MAPK is mediated via the endothelin-binding ET_A or ET_B receptor, we looked at the effect of endothelin A and B receptor antagonists, BQ610 and BQ788, respectively, on the tyrosine phosphorylation of ERK2, a hallmark of MAPK activation as assessed by Western blotting using a phosphotyrosine antibody following immunoprecipitation with anti-ERK2. The ETB receptor antagonist BQ788 completely abolishes endothelin-induced tyrosine phosphorylation of ERK2 whereas the ETA receptor antagonist BQ610 fails to inhibit the phosphorylation (Fig. 2), indicating that the activation of MAPK is mediated through the endothelin B receptor. The addition of 1 μg/ml pertussis toxin abolishes tyrosine phosphorylation of ERK2 (Fig. 3), showing that the Gi protein is associated with endothelin-induced signaling leading to the activation of MAP kinase. Similarly, the phospholipase C inhibitor, U73122, down-regulates tyrosine phosphorylation of ERK2 in a dose-dependent manner (Fig. 4), indicating that phospholipase C is also involved in the activation of MAPK during the intracellular signaling initiated by ET and ET_A receptor binding. The addition of the calcium chelator, BAPTA, has no effect on the phosphorylation of ERK2 (Fig. 5), showing there is no involvement of calcium mobilization in the activation of MAPK. In contrast, the PKC inhibitor, calphostine, abolishes the phosphorylation of ERK2 (Fig. 6), indicating that activation of PKC is required for the activation of MAPK.

Lack of Involvement of the PKC Pathway—The time course of the formation of IP3 following SCF and ET-1 treatment (Fig. 7) revealed that there is no difference in the level of IP3 following treatment with ET-1 + SCF or ET-1 alone, indicating no synergy occurring in the PKC pathway.

Synergistic Effect on the Pathway of MAPK Including Raf-1 and MEK—In the evaluation of tyrosine phosphorylation of ERK-1 and -2, an indicator of MAPK activation, as assessed by Western blotting using phosphotyrosine antibodies following immunoprecipitation with anti-ERK-1 and -2, we found that there is a synergistic effect on tyrosine phosphorylation in ET-1 + SCF-treated NHMC compared with ET-1 or SCF alone. There were increases both in the duration and intensity of tyrosine phosphorylation, which reached a peak within 10 min after stimulation (Fig. 8). In the evaluation of serine phosphorylation of MEK, an indicator of MAPKK activation, as assessed by Western blotting using a phospho-specific MEK antibody, there is a synergistic effect in SCF + ET-1-treated melanocytes with a peak within 4 min after stimulation (Fig. 9). This indicates that the synergy in the activation of MAPKK precedes the observed synergy in MAPK activation in SCF + ET-1-treated NHMC. Analysis of Raf-1 activity, an indicator of MAPKKK, as assessed by the final phosphorylation of MBP, showed that 10 nM ET-1 significantly stimulates Raf-1 activity,
which indicates that Raf-1 is at least one convergence point from the PKC pathway to the MAPK pathway. Furthermore, there is a synergistic effect on Raf-1 activity in SCF + ET-1-treated melanocytes (Fig. 10), suggesting the possibility that the synergistic convergence between SCF and ET-1 signaling is located nearby Raf-1.

**The Stimulatory Effect on the Formation of an Shc-Grb2 Complex**—The mechanism of tyrosine kinase receptor-stimulated MAPK signaling involves the formation of complexes between the guanine nucleotide exchange protein SOS, and the SH2 and SH3 domain-containing adaptor protein Grb2 either with autophosphorylated growth factor receptors or another tyrosine-phosphorylated adaptor protein known as Shc. Since Shc involvement in this signaling pathway requires tyrosyl phosphorylation, we compared the effects of SCF only and SCF + ET-1 on tyrosyl phosphorylation of Shc proteins in NHMC. Western blotting with phosphotyrosine antibodies following immunoprecipitation with Shc antibodies (Fig. 11) demonstrates that stimulation with SCF + ET-1 induces a more rapid and a stronger tyrosyl phosphorylation of proteins corresponding to p52 (1.2-fold in densitometric intensity) and p66 Shc than is elicited with SCF alone, and that this reaches a maximum (p52: 2.0-fold in densitometric intensity) within 5 min after stimulation. Upon activation of tyrosine kinase receptors, tyrosine-phosphorylated Shc associates with Grb2 and the guanine nucleotide exchange factor SOS, thereby leading to Ras activation. Therefore, we next examined whether the association of tyrosine-phosphorylated Shc with Grb2 also becomes synergistically marked following treatment with SCF + ET-1 as compared with SCF only. As shown in Fig. 12, more distinct

**FIG. 5.** The calcium chelator, BAPTA, has no effect on the phosphorylation of ERK2. Following incubation with the intracellular calcium chelator, BAPTA-AM (at 30 μM), human melanocytes were treated with 10 nM ET-1 and 5 min later were harvested and solubilized. The activation of MAPK was evaluated by measuring the tyrosine phosphorylation of ERK2 using Western immunoblotting as detailed under “Experimental Procedures.”

**FIG. 6.** Calphostine abolishes the phosphorylation of ERK2. Following incubation for 1 h with calphostine (at 3 μM), human melanocytes were treated with 10 nM ET-1 and 5 min later were harvested and solubilized. The activation of MAPK was evaluated by measuring the tyrosine phosphorylation of ERK2 as detailed under “Experimental Procedures.”

**FIG. 7.** No involvement of the PKC pathway in the synergism of ET-1 and SCF. Human melanocytes were stimulated with 10 nM ET-1 and/or 10 nM SCF. Levels of IP₃ were assayed at the indicated times as described under “Experimental Procedures.”

**FIG. 8.** Synergistic effect on tyrosine phosphorylation of ERK1/2. Human melanocytes were stimulated with 10 nM ET-1 and/or 10 nM SCF. The activation of MAPK was evaluated by Western immunoblotting using phospho-specific MAPK antibody as detailed under “Experimental Procedures.”

**FIG. 9.** Synergistic effect on serine phosphorylation of MEK1/2. Human melanocytes were stimulated with 10 nM ET-1 and/or 10 nM SCF. The activation of MEK was evaluated by measuring the serine phosphorylation of MEK at the indicated times by Western immunoblotting using phospho-specific MEK1/2 antibody as detailed under “Experimental Procedures.”
bands corresponding to p52 and p66 Shc are observed in immunoprecipitates from SCF + ET-1-treated melanocytes than from a single SCF stimulation. This shows that upon SCF + ET-1 stimulation, Grb2 associates more strongly in a complex with increased amounts of tyrosyl-phosphorylated Shc than it does upon a single SCF stimulation.

The Stimulated Activation of c-kit by the Combination of ET-1 and SCF—Finally, we examined whether the tyrosine phosphorylation of c-kit initiated by SCF binding to the c-kit receptor is stimulated by the concomitant addition of ET-1. Time course experiments assessed by Western blotting (Fig. 13) reveal a rapid and more marked tyrosine phosphorylation of c-kit, which reaches a maximum within 5 min after stimulation, with SCF + ET-1 compared with SCF only. In contrast, a single ET-1 stimulation does not elicit any tyrosine phosphorylation of c-kit, a finding which strongly suggests that ET-1 and ETB receptor binding initiated signaling stimulates tyrosine phosphorylation of c-kit only under conditions where c-kit is activated by SCF binding to its receptor.

DISCUSSION

In human melanocytes, it is well established that, after binding to its receptor, ET-1 triggers hydrolysis of polyphosphoinositide, which generates IP3 and diacylglycerol, mobilizing intracellular Ca2+ and activating PKC, respectively, which then stimulate proliferation and melanization (2, 16). In addition to the PKC pathway, ET has recently been shown to also activate the MAPK cascade (38) and G protein-coupled receptors (39), in relation to melanosis, respectively. Therefore, it is of interest to determine which signaling pathways are responsible for this synergistic effect. At first, we assumed that the convergence between ET-1 and SCF signaling occurs at the activation of MAPK, because we had previously found that ET-1-induced stimulation of melanization and proliferation in NHMC is associated with the activation of MAPK, a pathway very similar to that mediated by SCF (21–23). In this connection, Western blotting using tyrosyl MAPK antibodies demonstrates that the activation of MAPK occurs synergistically between SCF and ET-1-initiated signaling. Similar synergisms to the activation of MAPK have been reported between ET-1 and angiotensin II in cultured cardiomyocytes (30) and between SCF and erythropoietin in human erythroid colony-forming cells (39), in relation to mechanical stress-induced cardiac hypertrophy and expanded erythropoiesis, respectively.

Since MAPK activation is generally accompanied by the prior sequential activation of MEK and Raf-1 (40), we determined whether the synergism in the activation of MAPK is reflected by the synergistic activation of MEK and Raf-1. Related experiments using Western blotting and kinase assays revealed that there is a sequential synergistic activation in the MAPK cascade consisting of Raf-1, MEKK, MEK, and MAPK. This suggests that the convergence point between ET-1 and SCF-initiated signaling is located upstream of Raf-1.

As we confirmed a role for Raf-1 in the cross-talk mechanism between ET-1-associated PKC and SCF-associated tyrosine kinase pathways, we next determined signaling mechanisms leading to the synergistic Raf-1 activation by examining the influence of SCF on the ET-1-dependent PKC pathway. The formation of IP3 following stimulation is a hallmark for evaluation of PKC activation because IP3 and the protein kinase C activator, diacylglycerol, are simultaneously generated at an equimolar ratios. The time course of formation of IP3 following SCF and ET-1 treatment revealed that there is no difference in
the raised level of IP₃ induced by ET-1 + SCF or ET-1 alone, indicating no synergy occurs in the PKC pathway.

The mechanism of tyrosine kinase receptor-stimulated mitogenic signaling involves the formation between complexes of the guanine nucleotide exchange protein SOS, and the SH2 and SH3 domain-containing adaptor protein Grb2 with another tyrosine-phosphorylated adaptor protein Shc (21–23). Recent studies have shown that some G protein-coupled receptors utilize the same effectors as the tyrosine kinase receptor pathway (e.g. Shc-Grb-SOS), resulting in Ras and MAPK activation (41–43). However, it has been suggested that the pertussis toxin-sensitive G₁-coupled receptors utilize a pathway that induces Ras activation in a PKC-independent manner (44, 45). In this study, the synergism between ET-1 and SCF was found to be accompanied by synergistic tyrosyl phosphorylation of proteins corresponding to p52 and p66 Shc, leading to synergistic

**Fig. 11.** Synergistic effect on tyrosyl phosphorylation of proteins corresponding to p52 and p66 Shc. A. Immunoprecipitation and Western immunoblotting. B. Densitometric analysis. Human melanocytes were stimulated for the indicated times with 10 nM ET-1 and/or 10 nM SCF. The tyrosine phosphorylation of immunoprecipitated Shc was evaluated by Western immunoblotting as detailed under “Experimental Procedures.” Briefly, cell extract containing 0.5 mg of protein/sample was incubated with 4 μg of Shc antibody. Immunocomplexes were precipitated with protein G-Sepharose, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Blots were immunodetected with antiphosphotyrosine antibodies (1:1000). Similar results were obtained three times. IP, immunoprecipitation; WB, Western immunoblotting.

**Fig. 12.** Synergistic effect on the association of tyrosine-phosphorylated Shc with Grb2. Human melanocytes were stimulated for 2 min with 10 nM ET-1 and/or 10 nM SCF. The association of phosphorylated Shc and Grb2 was evaluated by immunoprecipitation with Shc or Grb2 antibodies, followed by Western immunoblotting as detailed under “Experimental Procedures.” IP, immunoprecipitation; WB, Western immunoblotting.
association of tyrosine-phosphorylated Shc with Grb2. It has also been shown that ET-1 signaling through heterotrimeric G protein-coupled receptors stimulates MAPK activity in primary cultures of astrocytes (46) via an increase in the tyrosine phosphorylation of Shc, which is followed by its stable association with Grb2 (31). Those studies suggested that ET-1-induced MAPK activation is a G protein-coupled pathway that involves Shc, Grb2, and probably Raf-1. Thus, the Shc-Grb2 complex may be involved in activation of the MAPK pathway, not only by several receptor tyrosine kinases but also by heterotrimeric G protein-coupled receptors, such as ET-1 receptors. In contrast to those studies using astrocytes, our study using human melanocytes showed that ET-1 does not stimulate tyrosine phosphorylation of Shc and its association with Grb2 even at its mitogenic and melanogenic concentrations. Interestingly, Western blotting analysis of tyrosine phosphorylation of c-kit upstream of Shc-Grb2 association revealed that this synergistic activation with adaptor molecules is initiated by the synergistic tyrosine phosphorylation of the SCF receptor, c-kit. Again, it should be noted that in human melanocytes the combination of ET-1 and SCF (but not ET-1 alone) enhances tyrosine phosphorylation of c-kit. A similar activation of receptor tyrosine kinases through intracellular signal cross-talk with ET-1-associated G-protein-coupled receptors has been documented for the epidermal growth factor receptor of Rat-1 cells (47) and of smooth muscle cells (48) in which only ET-1 can stimulate MAPK activation although the detailed mechanism is not clear.

In conclusion, the sum of the above findings indicates that synergistic cross-talk between SCF and ET-1 signaling is initiated through the pathway of tyrosine phosphorylation of c-kit. This results in the synergistically enhanced formation of the Ras/Raf-1/MAPK loop. ET-1 associated activation of PKC probably plays a role in the enhanced tyrosine phosphorylation of c-kit although the detailed mechanism is not clear. Thus, our results demonstrate a role for the c-kit tyrosine kinase receptor as a downstream mediator in synergistic mitogenic signaling induced by ET-1 + SCF and suggest a ligand-independent mechanism for c-kit activation through a synergistic intracellular signal cross-talk.

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FIG. 13. Synergistic effect on tyrosine phosphorylation of c-kit. Human melanocytes were stimulated for 2 or 5 min with 10 nM ET-1 and/or 10 nM SCF. c-kit proteins were extracted in RIPA buffer, immunoprecipitated with c-kit antibody and separated by SDS-PAGE. The phosphorylation of c-kit was detected using phosphorylated tyrosine antibody, as detailed under “Experimental Procedures.” IP, immunoprecipitation.
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