Plasma membrane protrusion is fundamental to cell motility, but its regulation by the extracellular environment is not well elucidated. We have quantified lamellipodial protrusion dynamics in human vascular smooth muscle cells exposed to fibroblast growth factor 2 (FGF-2) and type I collagen, two distinct ligands presented to vascular cells during arterial remodeling. Video microscopy revealed that FGF-2 stimulated a modest increase in lamellipodial protrusion rate that peaked within 15 min. This response was associated with immediate but transient activation of Rac1 and was inhibited in cells infected with retrovirus containing cDNA encoding dominant-negative Rac1. A 1-h exposure to FGF-2 also set up a second phase of more striking lamellipodial protrusion evident at 24–36 h. This delayed response was most pronounced when cells were on type I collagen and was associated with FGF-2-induced expression of collagenase-1 that localized to the edge of protruding lamellipodia. Moreover, late membrane protrusion was inhibited when cells were on collagenase-resistant type I collagen, implicating degraded collagen as a mediator. For cells on collagen, the immediate activation of Rac1 by FGF-2 was followed by a sustained wave of Rac1 activation that was inhibited when cleavage of the collagen triple helix was prevented and also by blockade of αvβ3 integrin. We conclude that lamellipodial protrusion in smooth muscle cells can be regulated by waves of Rac1 activation, corresponding to the sequential presentation of FGF-2 and remodeled collagen. The findings thus reveal a previously unrecognized level of coordination among extracellular input that enables cells to maintain protrusive activity over prolonged periods.

In the mature vasculature, smooth muscle cells (SMCs) serve primarily as units of contraction. However, vascular SMCs also have the capacity to migrate and do so during vascular remodeling and disease. In these circumstances, SMCs migrate to regions in the vessel wall that have been damaged, for example by inflammation, where they replicate and elaborate the extracellular matrix (ECM). Although this process is designed to be reparative, it can also be deleterious leading to narrowing of the vessel lumen, restricted blood flow, and organ ischemia.

SMC migration, like that of other cells, proceeds in a cycle that begins with the formation and protrusion of veil-like structures at the leading edge of the cell known as lamellipodia. Lamellipodia can stably attach to the ECM substrate, which enables traction forces to be generated to move the cell body forward. The trailing aspect of the cell then retracts to complete the cycle. Although all components of the migration cycle are necessary for cell translocation, protrusion of plasma membrane effectively leads the cell to its new location and is thus deemed to represent the essence of cell motility.

Intensive investigation in recent years has elucidated the mechanistic basis of lamellipodial protrusion as well as the signaling paths that impinge on the protrusion machinery (1, 2). Central to the process is a dynamic network of branching actin filaments that serve to push membrane forward. Each new branch of actin in the lamellipodial network forms on the side of a parent filament, under the control of the Arp 2/3 complex of proteins. Assembly of the Arp 2/3 complex is activated by the Wiskott-Aldrich syndrome protein and related proteins. Rho family GTPases, in turn, activate the Wiskott-Aldrich syndrome proteins. Of the Rho GTPases, Rac1 is particularly important to lamellipodial protrusion because it can effectively switch the lamellipodial ruffling process on or off, depending on its state of activation (3–5).

In contrast to growing data on the intracellular events underlying lamellipodial protrusion, less is known about the environmental signals that regulate these events. One extracellular factor that is known to generally influence SMC migration is fibroblast growth factor-2 (FGF-2, basic FGF) (6, 7). FGF-2 is liberated to act on SMCs following vascular injury and can stimulate SMCs to crawl from the medial vascular layer into the intimal layer (6, 8). We have observed that SMC migration is particularly enhanced by FGF-2 when cells are on a substrate of type I collagen (7). This apparent synergy between FGF-2 and type I collagen is noteworthy given the abundance of type I collagen fibrils in the interstitium of the vessel wall and the consequent need for migrating SMCs to navigate through this network.

The basic mechanism by which FGF-2 promotes SMC migra-
tion is not fully defined, but current evidence suggests that FGF-2 alters the interplay between SMCs and the surrounding ECM (9). In keeping with this concept, FGF-2 is one of a limited number of factors that can induce the expression of collagenase-1 in SMCs (10), which could effectively clear a path for SMC migration. In addition, we recently determined that FGF-2-induced expression of collagenase-1 enabled migrating SMCs to efficiently release their tail from the collagen substrate, which allowed the tail to smoothly retract into the cell body (11). Thus, FGF-2-induced type I collagen degradation appears to regulate the final, rear retraction phase of the locomotory cycle. Whether or not FGF-2, type I collagen, or their combination plays a more direct role in cell migration by driving lamellipodial protrusion is unknown. Protrusive processes at the cell front are fundamentally distinct from retraction events at the cell rear, yet these two processes must be coordinated for effective translocation of the cell. It is an intriguing question, therefore, whether the same environmental stimuli can orchestrate both forward protrusion and also rear retraction of plasma membrane and, if so, how this might occur.

To evaluate the impact of FGF-2 on plasma membrane protrusion in SMCs, we undertook video microscopic analyses of lamellipodial activity in primary human vascular SMCs. We provide evidence that lamellipodial protrusion is regulated by two temporally and mechanistically distinct phases. Exposure of SMCs to FGF-2 resulted in immediate but transient activation of Rac1 and transient protrusion of plasma membrane. This was followed by a prolonged phase of Rac1 activation and lamellipodial activity that was particularly striking when the cells were on a type I collagen substrate. This second wave of Rac1-mediated protrusion of plasma membrane was associated with collagenase-1 expression at the edge of protruding lamellipodia. Moreover, the second wave was dependent on type I collagen cleavage and signaling through α5β1 integrin. The findings suggest a model whereby environmental cues are dynamically coordinated to generate a sequential activation system for Rac1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Primary cultures of human vascular SMCs were established by explant outgrowth of unused segments of internal thoracic artery retrieved at the time of coronary artery bypass surgery (12). SMCs were studied in the second to fourth subcultures, and their identity was confirmed morphologically and by positive immunostaining for smooth muscle α-actin (1A4; Dako). The cells were grown in M199 supplemented with 10% FBS and antibiotics and plated on substrates of human fibronectin (100 μg/ml), rat tail collagen (100 μg/ml), or mouse tail collagen (100 μg/ml). Fibronectin was purified from human plasma by gelatin-Sepharose chromatography, as described (13). Rat and mouse tail collagen (primarily type I collagen) were isolated by solubilization in acetic acid, as described (14, 15). Mutant, collagenase-resistant collagen was prepared from tails of mice (B6,129S-Col1a1tm1Tch; Jackson Laboratory, Bar Harbor, ME) bearing targeted substitutions of 3 amino acids at the collagenase cleavage site of the α1(I) collagen chain (16, 17). These mutations render the triple helical domain of type I collagen resistant to cleavage by interstitial collagenases. Monoclonal anti-α5β1 integrin antibody (LM609) was purchased from Chemicon, and nonspecific control mouse IgG was obtained from the P3 cell line (ATCC).

**Generation of Recombinant Retrovirus and Infection of Human SMCs**—A retroviral gene delivery system (18) was utilized to generate human primary SMCs expressing mutant Rac1. cDNA fragments encoding dominant-negative (T17N/Rac) or constitutively active (G12VRac) Rac1 (19) (Guthrie cDNA Resource Center, Sayre, PA) were cloned into the bicistronic vector pRES2 (Clontech) with EGFP distal to the internal ribosome entry site. The bicistronic cassette was excised and cloned into pLNCX2 (Clontech). Retroviral delivery constructs were introduced into the Phoenix-ampphotropic retrovirus packaging/producing cell line (kindly provided by Dr. G. P. Nolan, Stanford University Medical School, CA; distributed by ATCC) (20) by calcium phosphate-mediated transfection. The virus-containing culture supernatant was harvested 48–72 h later and, after centrifugation and filtration (0.45-μm pore filters), was added to proliferating human SMCs with 5 μg/ml Sequa-brene for 48 h. SMC infection efficiency was estimated at 40% based on EGFP fluorescence. The cells stably expressing mutant DNA constructs or EGFP alone were selected with 500 μg/ml G418 for 2 weeks. Expression of mutant constructs was confirmed by Western blot analysis.

**Time Lapse Videomicroscopy and Measurement of Lamellipodial Protrusion Rate**—SMC motility was evaluated using digital time lapse videomicroscopy (11, 21). The cells plated onto 35-mm culture dishes coated with human fibronectin, rat tail collagen, or mouse tail collagen were imaged with an inverted microscope (Axiovert S100; Carl Zeiss, Inc. Thornwood, NY) using Hoffman-modulated contrast optics. The images were digitally acquired every 10 s over a 5–20-min recording period, using a CCD video camera (Sony XC-7575CE) and time lapse software (Northern Eclipse, Empix Imaging, Inc.). Ambient temperature was maintained at 37 °C by mounting the culture dish in a temperature control cell (CC-100; 2020 Technology Inc. Wilmington, NC), and a stream of warmed (37 °C) and humidified air was delivered continuously over a culture lid to prevent condensation. To maintain physiologic pH in the room air, the cells were incubated in bicarbonate-reduced medium M199 with Hanks’ salts and 25 mM Hepes.

To measure membrane protrusion, images acquired either 10 or 30 s apart were digitally overlaid, and a Boolean difference operation was applied. Subtracting the first of the two images from the second yielded the area of membrane that had protruded during the time interval (see Fig. 1). The area of all cell segments that had protruded over the 10- or 30-s interval was expressed as a fraction of the total cell area, determined from the first of each of the paired images. This procedure was repeated for the entire stack of images acquired over a 5–20-min recording (i.e., 30–120 serial image pairs, when using a 10-s image acquisition interval). The protrusion values were averaged and expressed as a mean fractional protrusion rate.

Membrane protrusion rates were determined in SMCs exposed to FGF-2 (25 ng/ml) for the designated period of time. Prior to stimulation, SMCs were rendered quiescent by incubating in M199 with 1% FBS for 48 h. To assess the effects of a discrete pulse of FGF-2, FGF-2 was removed from the cultures after 30 min, 1 h, or 4 h. The cells were then acid-washed twice (50 mM glycine, pH 2.8, 150 mM NaCl, 1 mg/ml polyvinylpyrrolidone) to remove receptor-bound FGF-2. SMCs were then incubated with basal media (M199 with 1% FBS) until the 24-h time point, at which time the lamellipodial protrusion rate was quantified.
Immunohistochemistry—Expression of collagenase-1 in human SMCs was assessed in SMCs grown on collagen-coated coverslips and fixed with 4% paraformaldehyde. The cells were incubated with a monoclonal antibody against human collagenase-1 (Calbiochem, Cambridge, MA), and bound primary antibody was detected using biotin-labeled goat anti-mouse IgG and avidin-peroxidase and diaminobenzidine tetrahydrochloride chromogen.

cdc42, Rac1, and RhoA Activity Assays—To assay the activity of Rac1 and cdc42, SMC lysate was affinity-purified using a fragment of p21-activated kinase 1 (PAK1) expressed as a fusion protein with glutathione S-transferase (GST) (22). The assay exploits the fact that PAK1, a downstream effector of Rac1 and cdc42, binds the respective GTPase in only its activated, GTP-bound form. cDNA from the protein-binding domain of PAK1 (PBD, corresponding to amino acids 67–150) was amplified by PCR from human placental cDNA and cloned into the bacterial expression vector pGEX-2T. This construct was expressed in Escherichia coli, and bacteria were lysed by passing through a French pressure cell at 20,000 p.s.i. and mixing with 1% Triton X-100. The lysate was mixed with 300 μl of glutathione-Sepharose beads (Pharmacia Bioscience), and the PBD-GST beads mixture was suspended in 150 μl of interaction buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin).

After stimulating SMCs on the designated substrate with FGF-2, the cells were washed in ice-cold phosphate-buffered saline and lysed with cold interaction buffer. Lysate containing 200 μg of protein (Bio-Rad) was incubated with 20 μl of the PBD-GST bead mixture overnight at 4 °C. Affinity-purified proteins were separated by 15% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted using monoclonal antibodies against human Rac1 (BD Transduction Laboratories) or human cdc42 (Santa Cruz Biotechnology). Bound primary antibody was detected with anti-mouse peroxidase-conjugated IgG secondary antibody and chemiluminescence detection reagents (ECL; Roche Applied Science) and x-ray film (X-Omat LS; Eastman Kodak Co.) exposure. The bands were assessed by densitometry (ChemiDoc; Bio-Rad), and the amount of PBD-bound Rac1 was compared with total cellular Rac1, ascertained by simultaneously immunoblotting cell lysate that was not subjected it to affinity purification. RhoA activity was assessed in a manner similar to that for Rac1, except that SMCs were lysed using RIPA buffer, and GTP-bound RhoA was affinity-purified using a Rhotekin-GST fusion protein (cDNA kindly supplied by M. Schwartz, La Jolla, CA) expressed in E. coli, as described (23).
noblotting was performed using a monoclonal antibody against human RhoA (Santa Cruz Biotechnology).

RESULTS

FGF-2 Stimulates Plasma Membrane Protrusion of Human Vascular SMCs—Migrating human SMCs are spread, quasipolygonal cells that have one or more lamellipodial extensions. In the absence of a strong directional stimulus for SMC migration membrane activity is unpredictable; some lamellipodia protrude, whereas other extensions might cease ruffling and shrink. Therefore, to accurately assess membrane protrusion in SMCs, we undertook rapid acquisition time lapse microscopy and analyzed the entire cell periphery throughout the recording period. Images acquired either 10 or 30 s apart were paired and digitally overlaid, and the area of protruding regions were quantified by a Boolean difference operation (Fig. 1). The proportion of the cell that displayed protrusion was quantified in serial image pairs for the entire recording period, from which a mean fractional protrusion rate was determined.

To determine whether FGF-2 could acutely influence plasma membrane dynamics, membrane protrusion in individual human SMCs was tracked serially under resting conditions (M199 with 1% FBS), following the addition of 0.1% bovine serum albumin, and following the addition of FGF-2. As depicted in Fig. 2, there was a prompt increase in plasma membrane protrusion following delivery of FGF-2. Averaged data showed that within 5 min of administration of FGF-2, there was a 1.4-fold increase in the fractional membrane protrusion rate (p < 0.01). This response reached a peak 10–15 min after delivery of growth factor and declined thereafter (Fig. 2, D and E). The substrate on which SMCs were plated had no effect on this immediate protrusion response to FGF-2, with no differences observed between SMCs on type I collagen, fibronectin, or vitronectin (data not shown).

To translocate the distances required in vivo, the relatively slow moving SMCs can be expected to crawl for sustained periods. Therefore, we next evaluated plasma membrane dynamics 36 h after the addition of FGF-2 to the cultures. As shown in Fig. 3 (including the video files), at this time point there was a marked increase in lamellipodial activity that was more pronounced than that seen immediately following FGF-2 delivery. In addition, we observed that the magnitude of the late protrusion response depended on the ECM substrate. Fractional plasma membrane protrusion rate for FGF-2-treated SMCs on collagen was substantially higher than that for FGF-2-treated SMCs on fibronectin (9.8 ± 0.9 versus 3.3 ± 0.4% min⁻¹, p < 0.001). The greater protrusive response for FGF-2-treated SMCs on type I collagen versus fibronectin did not seem to be a function of differential base-line adhesive forces, because SMCs abundantly express both collagen and fibronectin receptors (7) and varying the concentration of fibronectin (1–200 μg/ml) did not yield a response to FGF-2 that was as great as that for SMCs on type I collagen (10 or 100 μg/ml) (data not shown). In summary, FGF-2 induces immediate lamellipodial protrusion of SMCs but also endows SMCs with a pronounced ability to ruffle and extend lamellipodia for a sustained period. The magnitude of the sustained response depends on the ECM environment, with an apparent interplay between FGF-2 and type I collagen.

FGF-2 Induces Collagenase-1 Expression at the Cell Edge and Stimulates Collagenolysis-dependent Lamellipodial Protrusion—To elucidate how type I collagen could potentiate membrane protrusion of SMCs incubated FGF-2, we considered the fact that FGF-2 is known to induce the expression of collagenase-1 (10). This raised the possibility that lamellipodial activity might be influenced by proteolytic alteration of the collagen substrate. To verify that collagenase-1 was expressed in FGF-2-treated SMCs and to localize the enzyme, SMCs were immunostained using a monoclonal antibody against collagenase-1. No immunoreactivity was evident in vehicle-treated, subconfluent SMCs on either type I collagen or fibronectin. However, after treatment with FGF-2, collagenase-1 immunoreactivity was observed at the cell body, along the length of the tail, and, interestingly, as discrete signals at the edge of the extending lamellipodia (Fig. 4).

To determine the functional consequences of collagenase action on lamellipodial dynamics, we analyzed membrane protrusion of SMCs plated on collagenase-resistant mutant collagen. This substrate was prepared from the tails of mice harboring a mutation in the col1A1 gene corresponding to the collagenase-1 cleavage site. This mutation renders the collagen triple helix resistant to cleavage at the rate-limiting cleavage site, three-quarters of the way along the length of the triple helix (16, 17). As illustrated in the figures and video files of Fig. 5, the ability of FGF-2 to stimulate late, sustained membrane protrusion was blunted when SMCs were plated on collagenase-resistant
mouse tail collagen. The mean lamellipodial protrusion rate of FGF-2-treated SMCs migrating on mutant collagen was 29% that of FGF-2-treated SMCs on wild-type mouse tail collagen (\(p < 0.01\)) and similar to the protrusion rates found for FGF-2-treated SMCs on fibronectin (Fig. 2). Thus, the capacity to proteolytically edit a type I collagen substrate substantially influences lamellipodial activity in SMCs.

Lamellipodial Protrusion in FGF-2-treated SMCs Requires Rac1—To determine whether the membrane protrusive effects of FGF-2 in SMCs required Rac1, we generated human SMCs expressing dominant-negative Rac1 (T17NRac1) or constitutively active Rac1 (G12VRac1). Human SMCs transfect poorly,
and transgene expression was therefore accomplished by infecting SMCs with retrovirus containing cDNA encoding either T17NRac1 or G12VRac1 together with EGFP cDNA in a bicistronic vector. Fig. 6 depicts the acute response to FGF-2 in control and Rac1-perturbed human SMCs. SMCs expressing dominant-negative Rac1 showed no increase in lamellipodial protrusion during the 20 min after the administration of FGF-2. In contrast, SMCs expressing constitutively active Rac1 displayed broad fan-like lamellipodia that could comprise over half of the membrane perimeter. These cells displayed an elevated membrane protrusion rate under basal conditions, and there was no increase in lamellipodial protrusion after the delivery of FGF-2.

Membrane protrusion was also analyzed in T17NRac1-SMCs and G12VRac1-SMCs that were treated chronically (36 h) with FGF-2. As shown in Fig. 6B, SMCs infected with retrovirus containing cDNA for EGFP alone demonstrated a late response to FGF-2 that was similar to that for noninfected, FGF-2-stimulated SMCs. That is, FGF-2 treatment increased membrane protrusion rate moderately for cells on fibronectin and substantially for SMCs on type I collagen and with a blunted response for SMCs on collagenase-resistant collagen. In contrast, SMCs expressing dominant-negative Rac1 showed no increase in plasma membrane protrusion after prolonged incubation with FGF-2. Lamellipodial protrusion of SMCs expressing constitutively active Rac1 mimicked that of SMCs on collagen treated chronically with FGF-2, and the lamellipodial protrusion rate was not further increased by chronic exposure to FGF-2. In addition, the heightened membrane protrusion rate in G12VRac1-SMCs persisted when these cells were on collagenase-resistant collagen. Thus, constitutive augmentation of Rac1-mediated signaling circumvented the inhibitory effect imposed by a collagen substrate that cannot be efficiently degraded.

**FGF-2 Activates Rac1 in a Biphasic and Substrate-dependent Manner**—To determine whether FGF-2 activates Rac1 and to elucidate the profile of such activation, Rho GTPase activity assays were performed on serum-deprived SMCs plated on collagen, fibronectin, or collagenase-resistant collagen, before and after FGF-2 stimulation. As illustrated in Fig. 7, the level of active, GTP-bound Rac1 was substantially influenced by FGF-2, but the profile of activation depended on the ECM substrate. For SMCs on fibronectin, Rac1 activation was detectable 30 s after FGF-2 delivery. The activation was transient, and the levels of GTP-bound Rac1 declined to basal levels 10–30 min after FGF-2 administration and remained low for the subsequent 36 h. For SMCs on collagen, there was no increase in plasma membrane protrusion after prolonged incubation with FGF-2. Lamellipodial protrusion of SMCs expressing constitutively active Rac1 mimicked that of SMCs on collagen treated chronically with FGF-2, and the lamellipodial protrusion rate was not further increased by chronic exposure to FGF-2. In addition, the heightened membrane protrusion rate in G12VRac1-SMCs persisted when these cells were on collagenase-resistant collagen. Thus, constitutive augmentation of Rac1-mediated signaling circumvented the inhibitory effect imposed by a collagen substrate that cannot be efficiently degraded.
collagen was 4.3-fold higher than that for simultaneously assayed SMCs on fibronectin (p < 0.01). SMCs not stimulated with FGF-2 showed no evidence for Rac1 activation at either early or late periods, regardless of the ECM substrate. Active cdc42 was detectable in SMCs on fibronectin or collagen under basal, unstimulated conditions. However, the level of cdc42 activity did not change following FGF-2 stimulation (Fig. 7B).

The abundance of active, GTP-bound RhoA was also not increased by FGF-2 (Fig. 7C). Thus, FGF-2 appears to selectively activate Rac1. Moreover, the profile of Rac1 activation is strongly influenced by the ECM substrate such that a second, prolonged wave of Rac1 activation occurs when FGF-2-treated SMCs are on type I collagen.

To determine whether this second wave of Rac1 activation was dependent on proteolysis of the collagen substrate, SMCs were studied when plated on collagenase-resistant mouse tail collagen. Although, biphasic activation of Rac1 in response to FGF-2 was still observed under these conditions, the second phase was substantially attenuated (Fig. 7). Averaged data from three experiments revealed that Rac1 activity at 36 h was reduced to 0.44 ± 0.07 that of cells on wild-type collagen (p < 0.01). Thus, the delayed phase of Rac1 activation requires, at least partly, that the underlying collagen substrate be remodeled by ¼–1/4 cleavage of the triple helix, implicating remodeled collagen as a mediator of Rac1 activation.

*Delayed Rac1 Activation and Lamellipodial Protrusion in FGF-2-treated SMCs Is Mediated by α3β1 Integrin*—We next sought to determine the receptor through which remodeled collagen mediated the delayed phase of membrane protrusion in FGF-2-treated SMCs. Cleavage and denaturation of type I collagen is known to liberate epitopes for α3β1 integrin that do not exist in native type I collagen (24), a process suggested to provide new signals to cells within a fibrillar collagen milieu (25). Moreover, the β1 integrin subunit has recently been implicated in Rac1 activation in osteoclasts (26). To determine whether membrane protrusion of SMCs on collagen was mediated by α3β1 integrin, SMCs were stimulated with FGF-2 in the presence of an α3β1 integrin-blocking monoclonal antibody or isotype-matched control antibody. As shown in Fig. 8, blockade of α3β1 integrin had no effect on the immediate proliferative response to FGF-2. However, the delayed response, assessed by quantifying membrane protrusion at 24 h, was significantly inhibited (p < 0.05). In addition, and consistent with this finding, FGF-2-mediated Rac1 activation at 24 h was significantly inhibited when SMCs were co-incubated with the anti-α3β1 integrin antibody (Fig. 8B).

FGF-2 Does Not Need to Be Present during the Second Phase of Rac1 Activation and Lamellipodial Protrusion—The finding of two distinct waves of Rac1 activation for cells on type I collagen, and the role of collagen remodeling in the second phase, raised the question as to whether FGF-2 needed to be present for both phases of Rac1 activation. To address this, we undertook FGF-2 washout experiments (schematically depicted in Fig. 9). Human SMCs on type I collagen were stimulated with FGF-2 for varying lengths of time (30 min to 24 h), after which the FGF-2-containing medium was removed, and cells were acid-washed to remove receptor-bound FGF-2. The cells were then maintained in culture with basal medium (M199 plus 1% FBS) until the 24-h mark, at which time SMCs were analyzed for membrane protrusion and Rac1 activation.

As shown in Fig. 9B, maximal lamellipodial protrusion rates at 24 h were observed with as little as 1 h of stimulation with FGF-2, i.e., 23 h prior to assessing protrusion. A similar profile was observed for Rac1 activation (Fig. 9C). The pronounced, late activation of Rac1 for SMCs on collagen was observed with as little as 30 min of prior exposure to FGF-2. Therefore, although FGF-2 was a prerequisite for the second wave of Rac1 activation and lamellipodial protrusion, the cell did not require direct stimulation by FGF-2 at the time the second phase was proceeding.

FGF-2-mediated Collagenase-1 Expression in SMCs Is Partly Mediated by Rac1—It has been shown that collagenase-1 expression in synovial fibroblasts can be mediated by activation of Rac1 (27). This raised the possibility that the immediate activation of Rac1 by FGF-2 might itself play a role in the subsequent, collagenase-1-dependent phase of Rac1 activation and membrane protrusion. To determine whether collagenase-1 expression in SMCs was mediated by Rac1, SMCs were infected with retrovirus containing cDNA encoding constitutively active (G12VRac1) or dominant-negative (T17NRac1) Rac1, and collagenase-1 protein was evaluated by Western blot analysis. As shown in Fig. 10, SMCs expressing constitutively active Rac1 showed modest expression of collagenase-1 in the absence of stimulation with FGF-2, whereas SMCs expressing vector alone or dominant-negative Rac1 showed no collagenase-1 expression. Stimulation of vector-infected SMCs with FGF-2 induced collagenase-1 production, and this response was blunted, although not eliminated, in SMCs expressing T17NRac1. The level of collagenase-1 generated when G12VRac1-SMCs were stimulated with FGF-2 was somewhat higher than that of untreated G12VRac1-SMCs and also higher than that of control SMCs stimulated with FGF-2. These find-
ings indicate that FGF-2-mediated collagenase-1 expression is partly, although not entirely, mediated by Rac1. Thus, the initial burst of Rac1 activity may be a component of the cascade leading to the second wave of Rac1 activity. However, Rac1 activation does not appear to be the sole mechanism by which FGF-2 induces collagenase-1 expression in SMCs, and other, Rac1-independent pathways are also likely involved.

DISCUSSION

In this study, we have established that lamellipodial protrusion of human vascular SMCs is regulated by the integrated actions of FGF-2 and type I collagen, two distinct ligands that are presented to SMCs under conditions of vascular remodeling. These environmental constituents orchestrate the protrusion of plasma membrane via a dynamic mechanism that entails both nongenomic and genomic pathways. Exposure of SMCs to FGF-2 leads to immediate activation of Rac1 and Rac1-mediated protrusion of lamellipodia. This nongenomic response to FGF-2 is followed by the induction of collagenase-1 expression, localization of collagenase-1 to lamellipodial membrane, and proteolysis of the type I collagen substrate. The remodeled collagen, in turn, mediates a second phase of Rac1 activation, via $\alpha_\beta_3$ integrin, that is sustained and associated with even more pronounced protrusion of the plasma membrane (Fig. 11).

The current report provides the first documentation that Rac1 can be activated within seconds of exposing cells to FGF-2. Interestingly, FGF-2 did not stimulate acute activation of either cdc42 or RhoA in SMCs, suggesting a selectivity in GTPase activation by FGF-2 and the absence of an interplay among of Rac1, cdc42, and RhoA activation, in contrast to that observed in activated Swiss 3T3 cells (28). The consequence of acute activation of Rac1 in SMCs by FGF-2 was a prompt reorganization of the lamellipodial cytoskeleton, evidenced by
the immediate augmentation in plasma membrane protrusion that could be blocked by dominant-negative Rac1. Notably, however, the protrusive response was relatively modest, and it subsided after 15 min. It seems unlikely therefore that acute Rac1 activation by FGF-2 would in itself, drive significant translocation of a crawling cell. However, in addition to stimulating immediate membrane protrusion, this initial exposure to FGF-2 initiated a cascade of events, including collagenase-1 expression and collagen remodeling, that subsequently orchestrated the second, more sustained wave of Rac1 activity.

The notion of staged actions of external stimuli has recently emerged for growth factor-induced mitogenesis. Mitogenic growth factor is needed at both early (priming) and late (completion) phases for NIH3T3 cells to traverse the cell cycle (29). It is intriguing to consider that a somewhat similar paradigm exists for the regulation of cell motility. Under the conditions studied here, the two phases of signal activation for lamellipodial protrusion were mediated by soluble (FGF-2) and insoluble (remodeled collagen) environmental constituents, respectively. This is as opposed to a single growth factor acting at different times. Indeed, FGF-2 did not need to be present during the second wave of Rac1 activation for this wave to proceed. Instead, a 1-h exposure of SMCs on type I collagen to FGF-2 was sufficient to enable the cells to vigorously protrude plasma membrane up to 36 h later. As noted, induction of collagenase-1 expression by FGF-2 was critical for the delayed phase of Rac1 activation and membrane protrusion. Interestingly, collagenase expression by FGF-2 was partly mediated by Rac1. This suggests that the initial activation of Rac1 by FGF-2 was functionally linked to the second wave of Rac1 activation. At the same time, collagenase-1 expression did not appear to be completely dependent on Rac1 activation, and other pathways are likely important. Moreover, although the two phases of Rac1 activation are clearly linked, the external factors that directly stimulated each of the two phases differed.

We previously established that a proportion of the collagenase-1 expressed in response to FGF-2 was bound to the plasma membrane (11). Binding of collagenase-1 to the cell surface has also recently been observed in keratinocytes (30). In the current study, we found that in FGF-2-treated SMCs, collagenase-1 was localized to the leading edge of lamellipodial protrusions. This finding is in keeping with, and provides an explanation for, our previous observation that degraded type I collagen could be found beneath the leading front of motile SMCs (11), and it supports the notion that membrane protrusion may be influenced by collagen processing. Moreover, the current finding that extension of lamellipodia was constrained when FGF-2-treated SMCs were on collagenase-resistant collagen strongly implicates degraded collagen as a requirement for the delayed phase of lamellipodial protrusion.

There may be at least two broad mechanisms by which collagen degradation could promote membrane protrusion at the leading edge of the cell. First, by degrading collagen, a neostubstrate is generated that effectively allows for adherent membrane to be mobilized for protrusion. Collagen that has been subjected to the ¾–¾ triple helix cleavage will unfold, and the resulting denatured collagen fragments have a lower binding capacity for α3β1 integrin than does native type I collagen (31). Unfolded type I collagen also manifests cryptic RGD sites, which serve as ligands for β3 integrins on the cell surface (24, 32). Because the strength of binding between denatured collagen and α3β1 integrin is less than that of collagen binding to α3β1 integrin, the lifting and extension of lamellipodial membrane may be favored over sessile attachment to the substrate. Degraded collagen fragments have also been found to stimulate calpain-mediated cleavage of pp125FAK, with an associated reduction in SM attachment (33). Calpain-mediated remodeling of the focal adhesion complex may thus be another pathway by which type I collagen cleavage functionally loosens the lamellipodia from its substrate.

However, in addition to liberating plasma membrane for protrusion, the current findings establish that degraded collagen also engages the actin-based machinery for plasma membrane protrusion by activating Rac1. On a collagenase-resistant collagen substrate, little Rac1 activation was observed. However, Rac1 was activated when collagen remodeling proceeded, and this activation was mediated by α3β1 integrin. It remains possible that other factors, perhaps acting in an autocrine fashion, may also participate in the second wave of Rac1 activation. A functional β3 integrin cytoplasmic domain has recently been shown to be required for growth factor-stimulated Rac1 activation in osteoclast precursors (26). Nevertheless, a shift from native collagen to degraded collagen appeared to serve as a necessary switch that opened a second pathway for Rac1-mediated signaling. Dissecting downstream elements of this pathway will be important, and previously defined events arising from integrin activation, such as those involving phosphatidylinositol 3-kinase, Cas, Crk, and Rac1-dependent guanine nucleotide exchange factors, would be candidates (34–36).
For effective cell translocation, membrane protrusion at the cell front must be tightly coordinated with membrane retraction at the rear. We previously found that FGF-2-induced collagenase-1 activity enabled the cell to efficiently release its trailing aspect from a type I collagen substrate. In the absence of collagen degradation, the adherent cell tail could effectively hold the cell back from forward translocation, whereas FGF-2-induced collagen degradation allowed for smooth retraction of the cell rear and thus enabled forward gliding of the cell (10, 11). In light of the current findings, it is apparent that FGF-2-mediated collagen degradation serves as an environmental control system for both forward protrusion and rear retraction, despite the opposing and spatially discrete nature of these two events. Previous studies have shown that FGF-2 exerts little, if any, chemotactic activity and thus does not stimulate the directed migration of SMCs (7, 37). It is unlikely therefore that FGF-2 has a prime role in initially orienting the morphological or functional polarization of migrating SMCs. However, once a front and back have been established, for example by a gradient of PDGF-BB, the actions afforded by FGF-2 and FGF-2-mediated collagen turnover appear to have a substantial impact over both protrusion and retraction. This multiplicity of consequences implies an inherent mechanism for maintaining synchrony between the front and back of a migrating cell.

Following injury to the artery wall, SMCs are liberated from their basement membrane enclosure, whereupon they migrate rapidly through the collagen-rich tunica media to enter the tunica intima (38). In view of the current data, we propose this rapid egress of SMCs out the medial compartment is driven, at least in part, by the combined exposure of SMCs to FGF-2 and to type I collagen. Once in the intima SMCs, while still exposed to FGF-2, will interact with a nascent ECM that is now poor in fibrillar collagen but rich in provisional ECM components, including fibronectin. SMC migration still proceeds during this latter phase of vascular remodeling, but it is less striking than in the initial days following vascular injury. Based on the current findings, we speculate that in the face of persistent FGF-2 exposure, the shift from a type I collagen environment to a provisional ECM environment provides a “slowing” signal for SMC migration, by virtue of reduced activation of Rac1 and less robust membrane protrusion.

In summary, lamellipodial protrusion in human SMCs can be regulated by waves of Rac1 activation, corresponding to the sequential presentation of FGF-2 and remodeled collagen. FGF-2 stimulates immediate and transient Rac1 activation but also initiates collagenase-1-mediated degradation of type I collagen, yielding a neosubstrate that mediates sustained activation of Rac1 via $\alpha_\text{IIb}\beta_3$ integrin. This dual activation system for Rac1 suggests a previously unrecognized level of coordination among extracellular input that enables SMCs to maintain lamellipodial protrusive activity over prolonged periods. Because FGF-2-mediated proteolysis of collagen also promotes the detachment of the rear of the cell from its substrate, the findings also suggest a system for coupling forward protrusion and rear release.

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