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Ectodomain shedding of TGF-α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades

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A variety of transmembrane proteins, such as transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α) and L-selectin, undergo shedding, i.e. cleavage of the ectodomain, resulting in release of a soluble protein. Although the physiological relevance of ectodomain shedding is well recognized, little is known about the signaling mechanisms activating this process. We show that growth factor activation of cell surface tyrosine kinase receptors induces ectodomain cleavage of transmembrane TGF-α through activation of the Erk MAP kinase signaling cascade without the need for new protein synthesis. In addition, expression of constitutively activated MEK1 or its downstream target Erk2 MAP kinase was sufficient to stimulate TGF-α shedding. The basal cleavage level in the absence of exogenous growth factor stimulation was due to p38 MAP kinase signaling. Accordingly, a constitutively activated MKK6, a p38 activator, activated TGF-α shedding in the absence of exogenous stimuli. In addition to TGF-α shedding, these mechanisms also mediate L-selectin and TNF-α cleavage. Thus, L-selectin shedding by neutrophils, induced by *N*-formylmethionyl-leucyl-phenylalanine, was strongly inhibited by inhibitors of Erk MAP kinase or p38 MAP kinase signaling. Our results indicate that activation of Erk and p38 signaling pathways may represent a general physiological mechanism to induce shedding of a variety of transmembrane proteins.

Keywords: ectodomain shedding/MAP kinase/L-selectin/transforming growth factor-α/tumor necrosis factor-α

Introduction

Ectodomain shedding represents an important and efficient strategy to regulate the activities of a variety of transmembrane proteins (Werb and Yan, 1998). In the case of transmembrane growth factors, such as transforming growth factor-α (TGF-α) or other members of the TGF-α family, ectodomain cleavage releases the soluble growth factor from the cell surface (Derynck, 1992; Massagué and Pandiella, 1993). Soluble TGF-α is then able to diffuse and activate the epidermal growth factor (EGF)/TGF-α receptor (EGFR) and to induce receptor down-regulation in cells at a distance from the site of TGF-α synthesis. In contrast, transmembrane TGF-α activates the receptor only on neighboring cells and may not induce receptor internalization (Brachmann *et al.*, 1989; Wong *et al.*, 1989). Shedding of soluble TGF-α is often enhanced in tumor cells (Derynck, 1992), suggesting that signaling pathways that are activated in transformed cells may induce TGF-α ectodomain cleavage. Such a potentially important role for shedding in tumor formation is supported further by a transgenic mouse model, showing that mice overexpressing cleavable, but not the uncleavable TGF-α have an increased incidence of tumor development (Sandgren *et al.*, 1990).

Shedding also plays critical roles in inflammation. For example, shedding of the extracellular domain of the adhesion protein L-selectin disrupts binding of leukocytes to endothelial cells and may prevent their recruitment to sites of inflammation (Kishimoto *et al.*, 1989; Butcher, 1992; Rosen and Bertozzi, 1994). Shedding of transmembrane cytokines, such as tumor necrosis factor-α (TNF-α) or related proteins, also has dramatic effects. For example, the consequent diffusion and systemic release of soluble TNF-α is thought to cause cachexia, which cannot be induced by transmembrane TNF-α (Tracey and Cerami, 1990).

Despite the importance of shedding, the signaling mechanisms controlling this process are largely unknown. Inhibitor studies and functional experiments have revealed that the cleavage is mediated by cell surface metalloproteases (Crowe *et al.*, 1995; Arribas *et al.*, 1996; Feehan *et al.*, 1996; Solomon *et al.*, 1997). Such studies have led to the molecular identification of TACE (TNF-α converting enzyme), a transmembrane metalloprotease belonging to the ADAM family of proteases, as the protease responsible for TNF-α ectodomain cleavage (Black *et al.*, 1997; Moss *et al.*, 1997). TACE knockout mice display a phenotype similar to TGF-α-defective mice, and their fibroblasts are defective in shedding of not only TNF-α but also other cell surface molecules such as TGF-α and L-selectin (Peschon *et al.*, 1998), suggesting that TACE may mediate ectodomain cleavage of various transmembrane proteins. However, the mechanisms that regulate the activity of TACE and consequent ectodomain shedding are unknown.

Studies with mutant CHO cell lines further support the notion that shedding of a large group of cell surface proteins is regulated by similar regulatory mechanisms (Arribas *et al.*, 1996). The phorbol ester phorbol 12-myristate-13-acetate (PMA) is often used experimentally to induce shedding, and protein kinase C activation has therefore been thought to regulate shedding (Arribas *et al.*, 1996; Black *et al.*, 1997; Peschon *et al.*, 1998). While this manuscript was under review, Gechtman *et al.* (1999) suggested that PMA-induced cleavage of HB-EGF occurs through MAP kinase activation. However, Izumi *et al.* (1998) showed that the PMA-activated protein kinase Cδ stimulates HB-EGF cleavage through its ability directly to phosphorylate MDC9, another ADAM protease. Thus,

whether and how protein kinase C acts under physiological and pathological conditions to induce shedding have not been established. Besides PMA, shedding of the ectodomain of transmembrane TGF-α can also be induced by serum (Pandiella and Massagué, 1991), and EGF also induces its ectodomain cleavage (Baselga *et al*., 1996), thus suggesting that other signaling pathways may also lead to TGF-α ectodomain shedding. Finally, *N*-formylmethionyl-leucyl-phenylalanine (fMLP), an inflammatory mediator, is a potent inducer of L-selectin shedding, thus implicating yet another signaling mechanism that leads to ectodomain shedding (Bochner and Sterbinsky, 1991).

The aim of the current study was to begin to understand the signaling mechanisms that result in activation of ectodomain shedding of transmembrane TGF-α. We provide evidence that mitogenic growth factors that act through cell surface tyrosine kinase receptors activate ectodomain shedding. The basal level of ectodomain shedding is mediated by the stress-activated p38 MAK kinase pathway, whereas the growth factor-induced increase in shedding is mediated by the Erk MAP kinase pathway. These mechanisms are not restricted to transmembrane TGF-α, but represent general mechanisms that also regulate ectodomain shedding of transmembrane TNF-α and L-selectin. Accordingly, fMLP-induced shedding of L-selectin by neutrophils is inhibited by selectively blocking these two MAP kinase pathways.

Results

Mitogenic growth factors induce TGF-α ectodomain shedding

To study the extracellular cleavage of transmembrane TGF-α, we developed an assay that allowed a convenient and quantitative assessment of TGF-α shedding over time following stimulation. This was achieved in transient transfection assays in CHO cells, which lack endogenous EGFR and transmembrane TGF-α (Bringman *et al*., 1987) and have only low levels of endogenous fibroblast growth factor receptors (FGFRs) and platelet-derived growth factor receptors (PDGFRs). Thus, following transfection of an expression plasmid for transmembrane TGF-α, the proteins were pulse-^{35}S-labeled, and the time-dependent disappearance of cell-associated ^{35}S-labeled TGF-α and appearance of secreted TGF-α were assessed by immunoprecipitation using a monoclonal antibody against the extracellular 50 amino acid TGF-α core sequence (Figure 1A). In the cell lysate, pulse-labeled TGF-α appeared as three forms with different mobilities, resulting from differential glycosylation and processing of transmembrane TGF-α (Bringman *et al*., 1987). The 21 kDa middle band corresponds to the pro-polypeptide, without N-glycosylation, whereas the 25 kDa larger form is derived from the middle form and has undergone N-glycosylation of the N-terminal propeptide. The 12 kDa small form is generated after the removal of the 20 amino acid, glycosylated prosequence and derives from the larger and the middle forms (Bringman *et al*., 1987). Without stimulation, the large and small forms underwent a gradual low level of ectodomain cleavage, which resulted in changes in the ratios of the different TGF-α forms and their gradual overall decrease in intensity (Figure 1A, left). Concomitantly, secreted TGF-α appeared in the medium as two species (Figure 1B, left), the predominant 50 amino acid form (6 kDa) and an 18 kDa form, which has retained the N-glycosylated prosequence (Bringman *et al*., 1987). Whereas the secreted forms can be visualized following SDS–PAGE (Figure 1B), the level of secreted ligand can also be quantitated by scintillation counting of the secreted and immunoprecipitated soluble TGF-α (Figure 1C). The detection of the secreted growth factor allowed a quantitative assessment of the transmembrane TGF-α cleavage, which correlated with the intensity

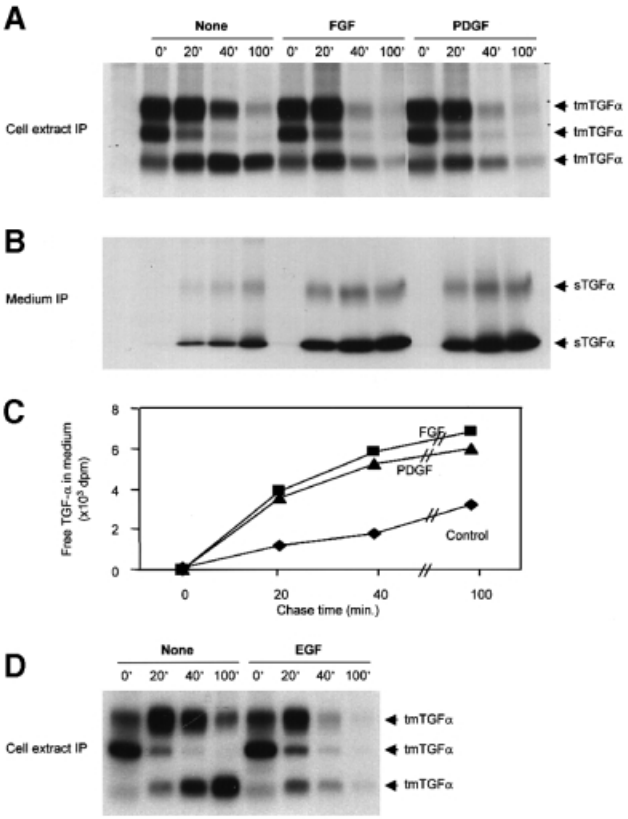

Fig. 1. Time-dependent stimulation of TGF-α ectodomain cleavage by growth factors. (**A**) Immunoprecipitation and SDS–PAGE of pulse-^{35}S-labeled transmembrane TGF-α in cell lysates at different times after addition of FGF or PDGF, or without growth factor addition (none). FGF and PDGF induce a more rapid processing and disappearance of the transmembrane forms (tmTGFα). The 21 kDa middle form contains the prosequence without N-glycosylation, which is processed into the larger 25 kDa form, i.e. the same polypeptide with the N-glycosylated prosequence, which in turn undergoes removal of the prosequence to yield the smaller 12 kDa form (Bringman *et al*., 1987). The left lane (unmarked) shows an anti-TGF-α immunoprecipitate from extract of cells transfected with a control expression vector without cDNA insert, chased for 0 min. (**B**) Immunoprecipitation and SDS–PAGE of pulse-^{35}S-labeled soluble TGF-α from the medium at different times after addition of FGF or PDGF, or without growth factor addition (none). Arrows point to the two forms of soluble TGF-α (sTGFα); the 6 kDa form corresponds to the 50 amino acid polypeptide, whereas the 18 kDa form corresponds to the glycosylated form with its prosequence (Bringman *et al*., 1997). The left lane (unmarked) shows an anti-TGF-α immunoprecipitate from the supernatant of cells transfected with a control expression vector without cDNA insert, chased for 100 min. (**C**) Quantitation by liquid scintillation counting of soluble ^{35}S-labeled, immunoprecipitated TGF-α, released by the cells treated by FGF or PDGF or untreated (control). (**D**) Induction of TGF-α cleavage by EGF in EGFR-expressing cells. Cell lysates were processed as in (A) and show the three transmembrane forms of TGF-α as specified in (A).

of the soluble TGF-α following gel electrophoresis. In addition, the increase of soluble TGF-α could be observed at 20 min of stimulation (Figure 1B and C), whereas a decrease of the transmembrane TGF-α was not evident until 40 min after stimulation (Figure 1A).

Using this assay, we determined that FGF and PDGF, two mitogenic growth factors that act through transmembrane tyrosine kinase receptors, stimulated the cleavage of transmembrane TGF-α and ectodomain release in a time-dependent manner. This was apparent not only by a faster disappearance of the large and small forms of transmembrane TGF-α (Figure 1A), but also by the increased level of soluble TGF-α, as assessed by both gel electrophoresis and scintillation counting (Figure 1B and C). Consistent with previous observations (Baselga *et al*., 1996), EGFR activation by EGF also resulted in increased cleavage of transmembrane TGF-α (Figure 1D). However, co-expression of the EGFR was required to assess the effect of EGF, since CHO cells lack endogenous EGFR (Livneh *et al*., 1986). Furthermore, the level of secreted TGF-α in the medium could not be determined accurately, since newly released TGF-α bound to the overexpressed EGFR, thus effectively diminishing the level of free TGF-α in the medium (Wang *et al*., 1998). Our experiments thus indicate that stimulation of these three tyrosine kinase receptors by mitogenic growth factors induced cleavage of transmembrane TGF-α and consequent release of soluble TGF-α.

FGF and PDGF stimulate TGF-α shedding through receptor protein tyrosine kinase activation

Most cellular responses to growth factors require the tyrosine kinase activity of their receptors (Ulrich and Schlessinger, 1990; van der Geer *et al*., 1994; Levitzki and Gazit, 1995). We therefore tested whether the kinase activity of the receptors was required to induce transmembrane TGF-α cleavage in response to FGF or PDGF. Thus, we tested the effects of the receptor-specific kinase inhibitors SU5402 and AG1296, which specifically prevent ligand-induced ATP hydrolysis by the stimulated FGFR or PDGFR, respectively (Levitzki and Gazit, 1995). Both inhibitors specifically decreased the ligand-induced stimulation of soluble TGF-α formation, although they did not significantly affect the level of transmembrane TGF-α (Figure 2A). Overexpression of cytoplasmically truncated versions of FGFR1 (Figure 2B) or of PDGFR-β (Figure 2C), which act as dominant-negative inhibitors of endogenous FGFR or PDGFR signaling, also inhibited the ability of FGF or PDGF, respectively, to stimulate transmembrane TGF-α cleavage. Thus, the enzymatic activities of FGFR and PDGFR are required to allow ligand-induced cleavage of transmembrane TGF-α.

Growth factor-induced TGF-α shedding does not require new protein synthesis

Growth factors regulate many cellular activities through their ability to activate signaling pathways that induce gene expression (Ulrich and Schlessinger, 1990; van der Geer *et al*., 1994; Iyer *et al*., 1999). However, the rapid increase of soluble TGF-α release following activation of the tyrosine kinase receptors (Figures 1 and 2) suggested that this effect may not depend on gene expression. Thus we tested whether new protein synthesis was required to induce transmembrane TGF-α cleavage. As shown in Figure 3, cycloheximide and emetine, two potent inhibitors of protein synthesis, did not affect the growth factor-induced cleavage of transmembrane TGF-α. Under these conditions, new protein synthesis was inhibited by 92% in the presence of either inhibitor, as assessed by incorporation of [^{35}S]methionine and [^{35}S]cysteine into trichloroacetic acid (TCA)-precipitable d.p.m. (data not shown). Our results therefore indicate that new protein synthesis was not required for this TGF-α ectodomain shedding.

MEK inhibition abolishes growth factor-stimulated TGF-α shedding

Several signaling pathways are activated upon growth factor-induced stimulation of tyrosine kinase receptors. Whereas the receptor-activated MAP kinase (Erk1/2) cascade mediated by Ras, Raf and MEK may have been best studied, receptor-induced activation of phosphatidylinositol (PI)-3 kinase, c-Src or phospholipase Cγ signaling regulates, either alone or in combination with MAP kinase signaling, the wide variety of cellular responses induced by growth factors (van der Geer *et al*., 1994; Pawson, 1995; Seger and Krebs, 1995; Denhardt, 1996). We therefore assessed which signaling pathway mediated the growth factor-induced TGF-α release using specific signaling inhibitors (Figure 4A). U73122 and LY294002, specific inhibitors of phospholipase Cγ and PI-3 kinase, respectively, did not affect the level of FGF- or PDGF-stimulated release of soluble TGF-α, even though they somewhat enhanced the level of transmembrane TGF-α, probably by inhibiting its turnover. These results suggest that the phospholipase Cγ and PI-3 kinase pathways do not regulate transmembrane TGF-α cleavage. In contrast, U0126, an inhibitor of MEK that activates Erk MAP kinase signaling, inhibited the FGF- or PDGF-induced generation of soluble TGF-α to slightly below the basal level (Figure 4A), strongly suggesting that the growth factor-induced cleavage of transmembrane TGF-α was due to activation of the MAP kinase pathway. A similar effect was also seen with PD098059, another specific inhibitor of MEK (data not shown). The decreased TGF-α cleavage by U0126 was also reflected in the increased levels of transmembrane TGF-α (Figure 4A), as expected, although an additional stabilizing effect on transmembrane TGF-α, e.g. by decreasing its turnover, cannot be excluded. Finally, treatment of cells with herbimycin A enhanced growth factor-induced transmembrane TGF-α cleavage, while it also slightly increased the transmembrane TGF-α levels (Figure 4A), suggesting that activation of c-Src (or another herbimycin-sensitive kinase) may down-regulate TGF-α ectodomain cleavage.

To investigate further the observation that the growth factor-induced cleavage of transmembrane TGF-α was mediated by the receptor-induced activation of the MAP kinase pathway, we overexpressed dominant-negative versions of MEK1 and/or MEK2 (Seger *et al*., 1994; Abbott and Holt, 1999), which are upstream activators of Erk MAP kinase signaling. Overexpression of either of these two mutant versions decreased the FGF-induced activation of the TGF-α release, albeit to a different extent (Figure 4B). The stronger inhibition by the dominant-negative MEK1 is consistent with its role as predominant activator of Erk MAP kinase in response to growth factors

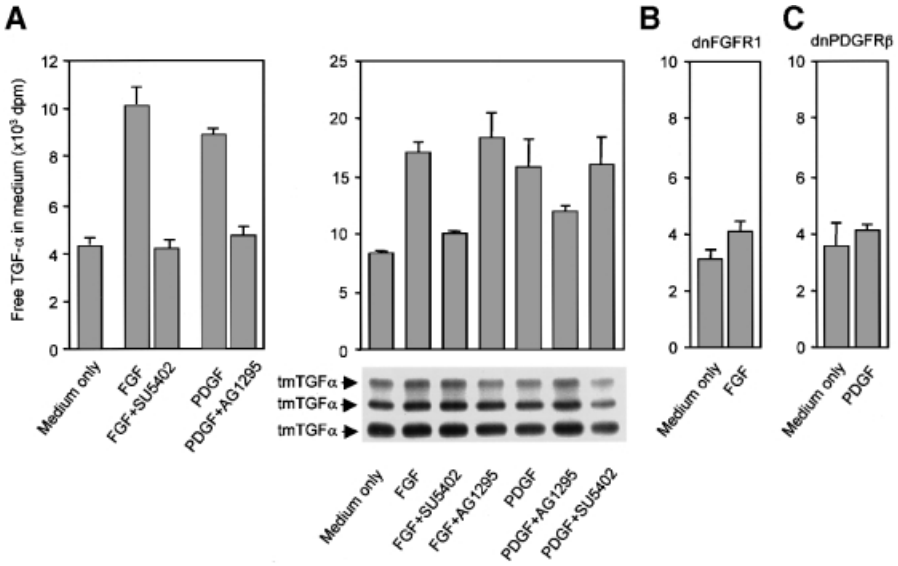

Fig. 2. Requirement for receptor tyrosine kinase activity for induction of TGF-α cleavage by FGF or PDGF. The soluble and transmembrane TGF-α were determined after 20 min of chase as in Figure 1A and C, respectively. Results of soluble TGF-α shown represent the average ± SD from duplicate samples. (**A**) Specific inhibition of FGF- or PDGF-induced cleavage by the FGFR inhibitor SU5402 or the PDGFR inhibitor AG1296, respectively. Results from two different experiments are shown. In the experiment on the right, a parallel determination of the amount of transmembrane TGF-α, as visualized by SDS–PAGE and autoradiography, is shown under the quantitation of soluble TGF-α. (**B**) Inhibition of FGF-induced TGF-α cleavage by expression of kinase-deficient FGFR1 (dnFGFR1). (**C**) Inhibition of PDGF-induced TGF-α cleavage by expression of cytoplasmically truncated PDGFRβ (dnPDGFRβ).

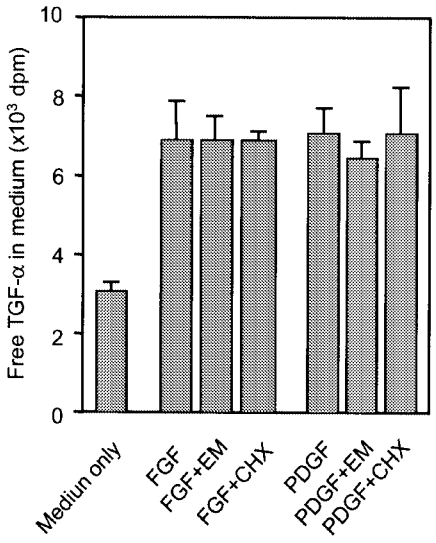

Fig. 3. Lack of effect of the protein synthesis inhibitors emetine (EM) or cycloheximide (CHX) on FGF- or PDGF-induced shedding of soluble TGF-α into the medium. The amounts of released TGF-α were determined after 20 min of chase in Figure 1C. Results shown represent the average ± SD from duplicate samples. In parallel experiments under the same conditions, EM and CHX inhibited new protein synthesis by 95%.

(Seger *et al*., 1994; Cohen, 1997). Overexpression of the dominant-negative mutants of both MEK1 and MEK2 together totally abolished the FGF-induced cleavage of transmembrane TGF-α (Figure 4B).

TGF-α shedding can be induced by constitutively activated MEK and Erk

Our observation that growth factor-induced transmembrane TGF-α shedding was abolished by inhibition of MEK1/2 activity strongly suggests that growth factor-induced cleavage of transmembrane TGF-α is due to receptor-activated MAP kinase signaling and predicted that constitutive activation of this signaling pathway would also induce transmembrane TGF-α cleavage. Thus, we tested the effect of a constitutively activated form of MEK1 or the MAP kinase Erk2. As shown in Figure 5, expression of either activated enzyme induced transmembrane TGF-α cleavage to an extent comparable to the growth factor-induced cleavage observed in, for example, Figure 2A. Overexpression of wild-type MEK1 did not stimulate transmembrane TGF-α cleavage, which is to be expected since activation of its kinase activity requires growth factor stimulation (Mansour *et al.*, 1994). Consistent with its specificity for MEK, the inhibitor U0126 inhibited the induction of transmembrane TGF-α cleavage by the constitutively activated form of MEK1, but not by the activated form of Erk2 (Figure 5).

PMA- and serum-induced TGF-α shedding is mediated by the MEK/Erk signaling cascade

The phorbol ester PMA and serum have previously been shown to induce TGF-α release (Pandiella and Massagué, 1991; Arribas *et al*., 1996; Peschon *et al*., 1998). We thus tested whether these activities of PMA and serum are also mediated through activation of MAP kinase signaling. As shown in Figure 6, the MEK inhibitor U0126 inhibited the TGF-α release induced by PMA or serum, suggesting that serum and PMA activate transmembrane TGF-α cleavage through their ability to activate the MAP kinase cascade.

The basal level of TGF-α shedding in CHO cells is mediated by the p38 MAP kinase signaling pathway

In the experiments described above, inhibition of the Erk MAP kinase pathway efficiently inhibited the growth factor-induced TGF-α release. However, this inhibition

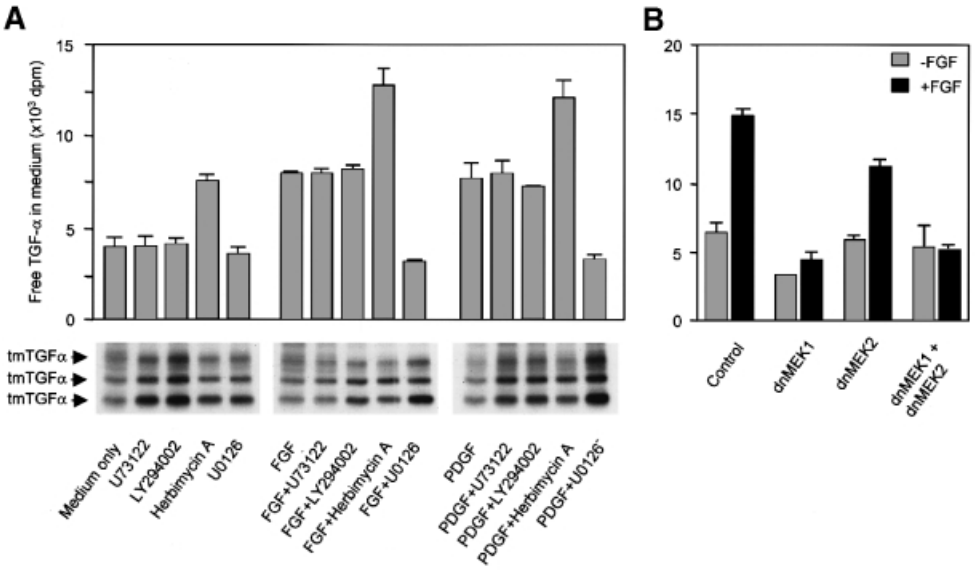

Fig. 4. Erk MAPK signaling mediates growth factor-induced TGF-α shedding. The soluble and transmembrane TGF-α were determined after 20 min of chase as in Figure 1C and A, respectively. Results of soluble TGF-α shown represent the average ± SD from duplicate samples. (**A**) Inhibition of FGF- and PDGF-activated TGF-α cleavage by the MEK inhibitor U0126, but not by U73122, LY294002 or herbimycin A. Inhibitors were added into chase medium with or without growth factors. A parallel determination of the amount of transmembrane TGF-α, as visualized by SDS–PAGE and autoradiography, is shown under the quantitation of soluble TGF-α. In the absence of growth factor, U73122 and LY294002 induced a slight increase in the levels of transmembrane TGF-α, but this did not result in enhanced levels of soluble TGF-α. U0126 inhibited the FGF- or PDGF-induced TGF-α release and this was reflected in the higher levels of transmembrane TGF-α. (**B**) Inhibition of FGF-induced TGF-α cleavage by expression of dominant-negative MEK1 (dnMEK1) or MEK2 (dnMEK2), or both.

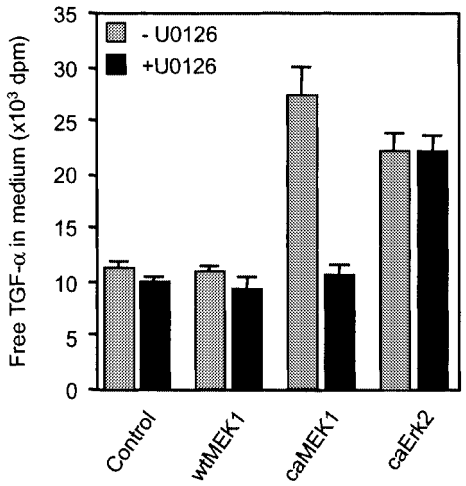

Fig. 5. Induction of TGF-α cleavage by expression of constitutively activated MEK1 (caMEK1) or Erk2 (caErk2). Cells co-transfected with the transmembrane TGF-α expression plasmid and vectors for wild-type (wt), caMEK1 or caErk2 were labeled for 2 h. Labeling medium was then collected and used for immunoprecipitation of TGF-α. TGF-α release was induced by both caMEK1 and caErk2, but only the caMEK1-induced release was inhibited by U0126, consistent with its specificity.

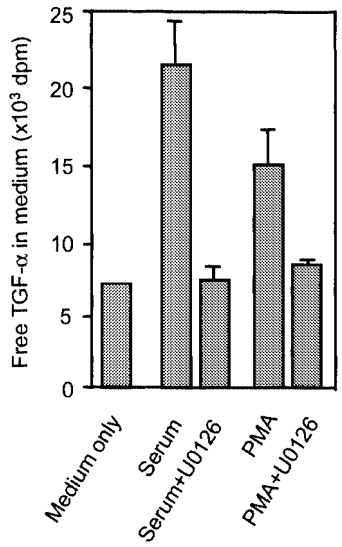

Fig. 6. Inhibition of serum- and PMA-induced TGF-α cleavage by the MEK inhibitor U0126. The amounts of released TGF-α were determined following 20 min of chase as in Figure 1C. Results shown represent averages ± SD from duplicate samples.

did not significantly affect the basal level of TGF-α cleavage in the absence of exogenous stimulation (Figures 4–6). These findings suggested that the basal level of transmembrane TGF-α cleavage might perhaps be due to a related, but different signaling pathway. We found that SB202190, a specific inhibitor of p38 MAP kinase, strongly decreased the basal level of TGF-α release, but did not qualitatively affect the ability of FGF or PDGF to induce transmembrane TGF-α cleavage (Figure 7A). Thus, FGF or PDGF treatment resulted in a 6- to 10-fold increase above the very low basal level in the presence of SB202190, yet the total amount of released TGF-α was still below the growth factor-induced level of soluble TGF-α in the absence of the inhibitor (Figure 7A). Consistent with this finding, constitutively activated MEK1 or Erk2 stimulated transmembrane TGF-α cleavage in the presence of SB202190 (data not shown). These results strongly suggest that activation of p38 MAP kinase controls the basal level of TGF-α release, without affecting its inducibility by growth factors, whereas the induction of the Erk MAP kinase pathway mediates the increase in cleavage following growth factor receptor activation. Consistent with the role of p38 MAP kinase signaling,

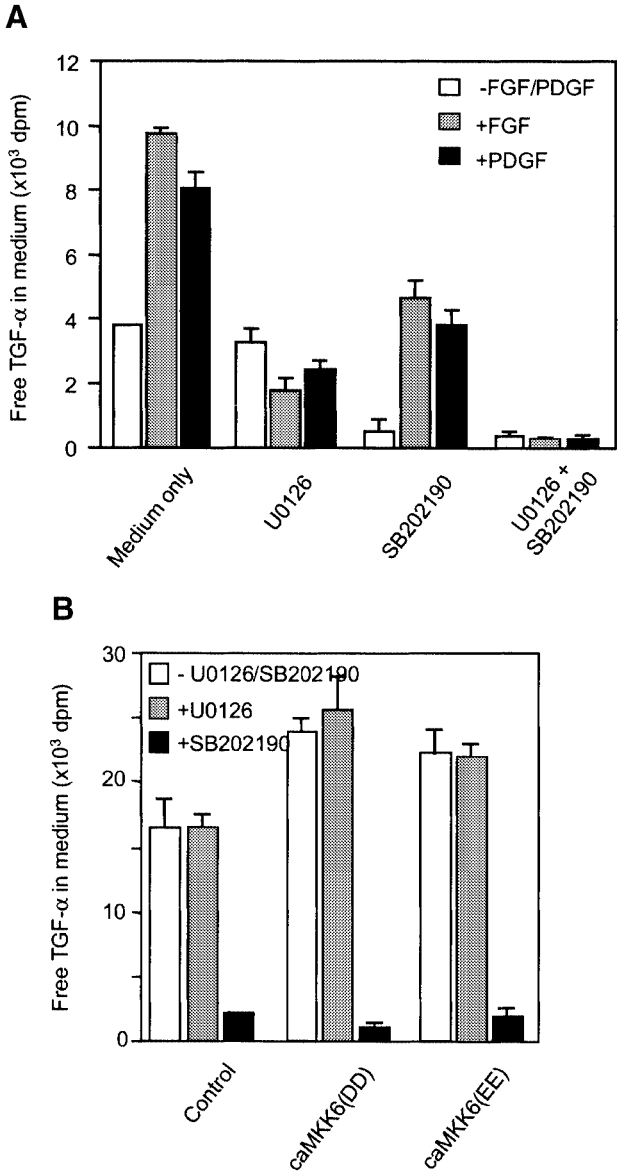

Fig. 7. p38 MAP kinase signaling mediates the basal level TGF-α cleavage in cell culture. The amounts of released TGF-α were determined following 20 min of chase as in Figure 1C. Results shown represent averages ± SD from duplicate samples. (**A**) Differential effects of U0126 and p38 MAP kinase inhibitor SB202190 on basal and growth factor-activated TGF-α cleavage. U0126 and/or SB202190 were present in the medium during the 2 h labeling. Cells were washed and chased with medium containing appropriate growth factors and inhibitors. The levels of free TGF-α were normalized against the level of transmembrane TGF-α at the beginning of the chase. (**B**) Induction of TGF-α cleavage by constitutively activated MKK6 mutants, caMKK6(DD) or caMKK6(EE). Cells co-transfected with TGF-α and the MKK6 vectors were labeled for 2 h. Labeling medium was collected and used directly for immunoprecipitation of TGF-α. The d.p.m. values of soluble TGF-α were normalized against the levels of transmembrane TGF-α immunoprecipitated from extracts of transfected cells that were labeled for 10 min.

overexpression of constitutively activated forms of MKK6, an upstream activator of p38 MAP kinase (Raingeaud *et al.*, 1996), resulted in enhanced TGF-α release in the absence of growth factor stimulation (Figure 7B). This enhancement was completely abolished by SB202190, but was not affected by U0126 (Figure 7B). Finally, the presence of both SB202190 and U0126 not only inhibited the basal level of TGF-α release, but also prevented its induction by FGF or PDGF, further illustrating the cooperativity of both signaling pathways (Figure 7A).

L-selectin and TNF-α ectodomain release is induced by the same MAP kinase signaling pathways as TGF-α shedding

Various other transmembrane proteins, such as L-selectin and TNF-α, also undergo proteolytic shedding of their ectodomains (Kishimoto *et al.*, 1989; Rosen and Bertozzi, 1994; Crow *et al.*, 1995; Arribas *et al.*, 1996; Solomon *et al.*, 1997), and, similarly to TGF-α, the cleavage of L-selectin or TNF-α can be induced by PMA (Arribas *et al.*, 1996; Peschon *et al.*, 1998). The increased ectodomain shedding of L-selectin or TNF-α in association with inflammation (Kishimoto *et al.*, 1989; Solomon *et al.*, 1997) raises the possibility that growth factors for immune cells or other activators of MAP kinase signaling might induce their shedding as well. The finding that a mutant cell line, deficient in transmembrane TGF-α cleavage, is also deficient in L-selectin and TNF-α shedding further suggests that the same regulatory mechanisms may underlie the cleavage of both proteins (Arribas *et al.*, 1996). Finally, the lack of PMA-induced transmembrane TGF-α or L-selectin cleavage in cells that are deficient in TACE (Peschon *et al.*, 1998) suggests that the same enzyme may regulate the cleavage of these different transmembrane proteins and thus may be subject to the same regulatory control.

We therefore evaluated whether the mechanisms that control TGF-α release, i.e. basal level of shedding through p38 MAP kinase signaling and growth factor-induced shedding through the Erk MAP kinase pathway, are general mechanisms to regulate cell surface protein shedding and thus induce L-selectin and TNF-α cleavage. As shown in Figure 8A, FGF or PDGF induced cleavage of transmembrane TNF-α, thus resulting in a decreased level of transmembrane TNF-α and an increased level of 17 kDa soluble TNF-α within 20 min of treatment. FGF and PDGF treatment also induced L-selectin shedding, thus resulting in decreased levels of fully glycosylated, 74 kDa transmembrane L-selectin and increased levels of both the 68 kDa soluble L-selectin and the corresponding 6 kDa cell-associated cytoplasmic tail (Figure 8B). The extent of cleavage in response to growth factors was similar to the induction in response to serum or the phorbol ester PMA (Figure 8A and B). The induction of L-selectin or TNF-α cleavage by all four stimuli was inhibited to approximately the basal level by U0126, the inhibitor of Erk MAP kinase signaling (Figure 8A and B). In addition, the constitutively active MEK1 and the constitutively active Erk2 MAP kinase induced cleavage of transmembrane TNF-α (Figure 8C) or L-selectin (Figure 8D) in the absence of exogenous growth factor stimulation. Thus, as in the case of transmembrane TGF-α cleavage, growth factor-activated tyrosine kinase receptors induce the cleavage, and this stimulation is mediated by the activated Erk MAP kinase signaling pathway. We also evaluated the role of p38 MAP kinase signaling in mediating the basal level of transmembrane TNF-α or L-selectin cleavage. Again, similarly to transmembrane TGF-α cleavage, the basal levels of transmembrane TNF-α (Figure 8E) or L-selectin (Figure 8F) cleavage were strongly inhibited

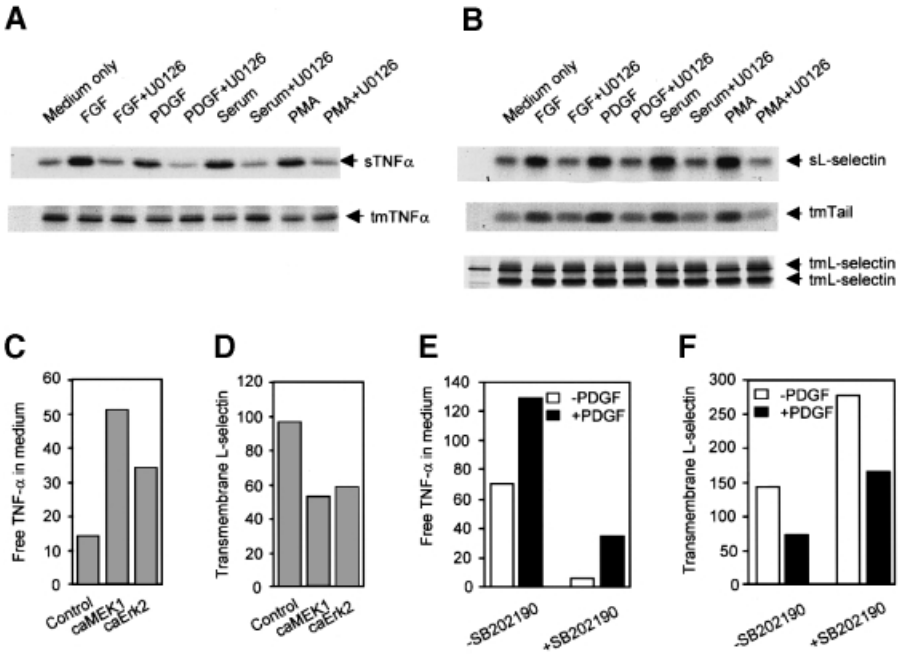

Fig. 8. Induction of TNF-α and L-selectin shedding by Erk and p38 MAP kinase pathways. (**A**) U0126 inhibits TNF-α cleavage induced by FGF, PDGF, serum or PMA. The amount of soluble TNF-α released into the medium (sTNFα) and transmembrane TNF-α (tmTNFα) at 20 min of chase was visualized by SDS–PAGE followed by autoradiography. The unmarked left lane in the gels shows control immunoprecipitates of chase medium or extract from cells transfected with the same expression vector but without cDNA insert. (**B**) U0126 inhibits L-selectin cleavage induced by FGF, PDGF, serum or PMA. The levels of 68 kDa soluble L-selectin (sL-selectin) and resulting cell-associated 6 kDa cleavage product, containing the transmembrane and cytoplasmic segments (tmTail), and the cell-associated 74 and 50 kDa L-selectin (tmL-selectin), at 10 min of chase, were visualized by SDS–PAGE followed by autoradiography. Only the fully glycosylated, 74 kDa tmL-selectin form is known to undergo shedding (Kahn *et al.*, 1994; Arribas *et al.*, 1996). The unmarked left lane in the gels shows control immunoprecipitates of chase medium or extract from cells transfected with the same expression vector but without cDNA insert. (**C**) Induction of TNF-α cleavage by constitutively activated (ca) MEK1 and Erk2, as assessed by densitometric scanning of the soluble TNF-α band on the gel [as shown in (A)]. Cells co-transfected with the TNF-α expression plasmid and vectors for caMEK1 or caErk2 were labeled for 2 h. Labeling medium was collected and used directly for immunoprecipitation of TNF-α. The densitometric values were corrected for transfection efficiency against the amount of transmembrane TNF-α precipitated from extract of transfected cells that were labeled for 10 min. (**D**) Stimulation of L-selectin cleavage by constitutively activated (ca) MEK1 and Erk2, as assessed by densitometric scanning of the 74 kDa tmL-selectin band on the gel [as shown in (B)]. Cells tranfected with an L-selectin plasmid were labeled for 2 h, and chased for 10 min in medium containing no stimuli. (**E**) The basal level, but not the growth factor-stimulated, TNF-α cleavage is inhibited by SB202190, as assessed by densitometric scanning of the soluble TNF-α band on the gel. SB202190 was present in medium during the 2 h labeling. Cells were washed and chased with medium containing the inhibitor with or without PDGF for 20 min. (**F**) SB202190 inhibits the basal, but not the growth factor-induced, L-selectin cleavage, as assessed by densitometric scanning of the 74 kDa tmL-selectin band on the gel. SB202190 was added into medium during the 2 h labeling. Cells were washed and chased with medium containing the inhibitor with or without PDGF for 10 min. Densitometric values shown are arbitrary.

by SB202190, the inhibitor of p38 MAP kinase signaling, but this inhibition did not affect the ability of growth factors to induce cleavage through the Erk MAP kinase signaling pathway.

fMLP-induced L-selectin shedding in neutrophils is inhibited by MEK and p38 MAPK inhibitors

A commonly used model system to study L-selectin shedding in a physiological context is to treat freshly isolated human neutrophil cells with the inflammatory mediator fMLP. fMLP treatment of neutrophils results in rapid shedding of L-selectin and, consequently, a rapid decline in the cell surface levels of L-selectin, which can be assessed by flow cytometry (Elbim *et al.*, 1994). This is illustrated in Figure 9 by the rapid shift of the fluorescence peak between the untreated and fMLP-treated cultures. To test the roles of the Erk MAP kinase and p38 MAP kinase signaling pathways, we tested the effects of U0126, the inhibitor of Erk MAP kinase signaling, or of SB202190, the inhibitor of p38 MAP kinase signaling, on fMLP-induced shedding of L-selectin. Both inhibitors strongly inhibited fMLP-induced L-selectin cleavage from neutrophils, therefore providing evidence that the two kinase pathways activate ectodomain shedding in response to an inflammatory mediator. The strong inhibition by either inhibitor individually strongly suggests a cooperative functional cross-talk between these two MAP kinase pathways. Such signaling cooperativity between MAP kinase pathways has been characterized previously in several experimental settings (Nagata *et al.*, 1998; Ogura and Kitamura, 1998).

Discussion

In this report, we have studied the physiological signaling mechanisms that lead to ectodomain shedding, using transmembrane TGF-α as the primary model. Several important observations have emerged from our studies. First, mitogenic stimulation through tyrosine kinase receptors activates ectodomain shedding. Secondly, induction of MAP kinase signaling, i.e. either the Erk MAP kinase pathway or the p38 MAP kinase pathway, or both, fully accounts for both the growth factor-induced and the basal level of shedding. Thirdly, the induction of shedding in

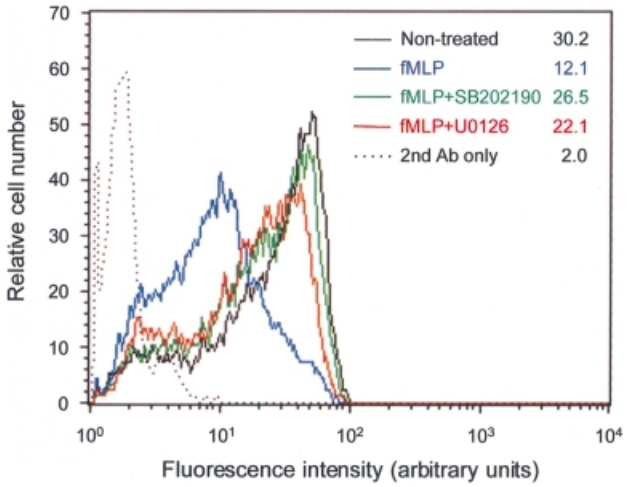

Fig. 9. Inhibition of fMLP-induced neutrophil L-selectin shedding by SB202190 and U0126. Freshly isolated human neutrophils were incubated in the presence or absence of inhibitors for 15 min, stimulated with 100 nM fMLP for 5 min, sequentially stained with DREG-56 and fluorescein-conjugated secondary antibody, and finally analyzed by flow cytometry. The mean fluorescence intensity for each sample is shown beside the key. The *y*-axis values for non-treated cells stained with only second antibody were scaled down by 50%.

response to MAP kinase signaling is a general mechanism. This mechanism not only mediates shedding in response to several inducers, including PMA and fMLP, but also regulates shedding of all three transmembrane proteins tested, i.e. TGF-α, TNF-α and L-selectin, and presumably many more.

The ability of growth factors to activate ectodomain shedding of transmembrane growth factors may have important physiological implications for the regulation of cell growth. Besides TGF-α, other members of this family, such as HB-EGF and amphiregulin, are also made as transmembrane proteins and undergo ectodomain shedding (Raab and Klagsbrun, 1997; Brown *et al*., 1998; Izumi *et al*., 1998). Growth factor stimulation of cells that produce these transmembrane growth factors, e.g. during the wound healing process, is likely to result in the release of soluble growth factors through ectodomain shedding. These soluble growth factors then have the ability to stimulate cells that would normally not be reached through cell–cell contact with the growth factor-producing cells. Thus, growth factor-induced shedding may provide a mechanism to amplify growth factor stimulation. In the case of TGF-α members, ectodomain shedding may be part of a positive feedback mechanism. Thus, activation of the EGFR by TGF-α (or related ligands) is known to induce TGF-α expression (Coffey *et al*., 1987) and this response would then be complemented further by the EGFR-induced ectodomain shedding of transmembrane TGF-α (Baselga *et al*., 1996; this report). Growth factor-induced shedding may also play a role in tumor development. Autocrine growth factor responsiveness contributes to cell transformation and tumor development, as has been well documented in the case of TGF-α (Derynck, 1992; Khazaie *et al*., 1993; Lee *et al*., 1995). Thus, growth factor-induced ectodomain shedding of transmembrane growth factors may amplify the autocrine or paracrine growth stimulation of tumor cells and in this way contribute to tumor development. The importance of ectodomain shedding in tumor development is supported by the observation that transgenic mice which overexpress wild-type transmembrane TGF-α develop carcinomas, whereas overexpression of a mutated version of transmembrane TGF-α that cannot undergo ectodomain cleavage does not result in tumor formation (Sandgren *et al*., 1990).

Following mitogen-induced activation of tyrosine kinase receptors, several signaling pathways are activated (van der Geer *et al*., 1994; Pawson, 1995; Denhardt, 1996). Among these, only the Erk MAP kinase signaling cascade acts as an effector pathway for growth factor-induced activation of the shedding metalloprotease(s) and consequent ectodomain release. The role of the Erk MAP kinase signaling cascade was identified through the use of constitutively activated MEK1 or Erk2, dominant-negative mutants of MEK1 and MEK2, and the highly specific MEK inhibitor U0126. Inhibition of this pathway fully repressed growth factor-induced ectodomain shedding. Importantly, activation of ectodomain shedding did not require new protein synthesis, possibly suggesting that the protease activation may depend on a critical phosphorylation event. The measurable basal level of ectodomain cleavage in culture allowed us to identify the p38 MAP kinase pathway as another signaling pathway that controls ectodomain shedding. As with the Erk MAP kinase pathway, the ability of this pathway to induce shedding was determined using a specific inhibitor of p38 MAP kinase, i.e. SB202190, and constitutively active versions of MKK6. Since this pathway is activated by stress signals and inflammatory mediators (Denhardt, 1996; Cohen, 1997), it is likely that this basal level of shedding is a result of cell culture conditions, rather than a reflection of a constitutive level of shedding under normal physiological conditions *in vivo*. Since c-Src activation can induce signaling by both the Erk and p38 MAP kinase pathways (Aikawa *et al*., 1997; Daulhac *et al*., 1999; Su *et al*., 1999), the ability of the Src kinase inhibitor herbimycin A to enhance ectodomain shedding, both in unstimulated and growth factor-stimulated cells, is surprising. We speculate that this effect was due to the inhibition of an as yet unknown kinase by herbimycin A. Interestingly, the enhanced shedding in the presence of herbimycin A could be inhibited by SB202190, but not by U0126 (data not shown). This herbimycin-sensitive kinase may therefore normally inhibit shedding by its ability to down-regulate the p38 MAP kinase signaling activity.

The combination of the Erk and p38 MAP kinase pathways fully accounts for the induction of ectodomain shedding in our experiments using different inducers. Growth factors that act through tyrosine kinase receptors induce Erk MAP kinase signaling, and inhibition of this pathway fully blocks growth factor-induced shedding. The basal shedding was mediated by the p38 MAP kinase pathway, and inhibition of this pathway did not affect growth factor-induced shedding qualitatively, but decreased the levels of both the basal and growth factor-induced shedding. Thus, both pathways appear to function additively in growth factor-induced shedding. In contrast to growth factor stimulation, the inflammatory peptide fMLP induces both the p38 and the Erk MAP kinase pathways. Whereas these two pathways function additively in growth factor-induced shedding, they seem to display

signaling cooperativity in fMLP-induced shedding. Indeed, inhibition of either pathway alone using U0126 or SB202190 resulted in a strong inhibition of L-selectin shedding. A similar type of signaling cooperativity between MAP kinase signaling pathways has been observed in the control of cell proliferation (Nagata *et al*., 1998). Finally, the induction of Erk MAP kinase signaling also accounts for the activation of ectodomain shedding by PMA, a potent activator of protein kinase C. This finding is in agreement with the very recent report of Gechtman *et al*. (1999). Thus, inhibition of Erk MAP kinase signaling blocked PMA-induced shedding, whereas inhibition of p38 MAP kinase signaling blocked the basal level, similarly to what we observed in growth factor-induced shedding. Since protein kinase C activation has been shown to induce Erk MAP kinase signaling (Morrison *et al*., 1996; Marais *et al*., 1998; Schönwasser *et al*., 1998), we conclude, in agreement with Gechtman *et al*. (1999), that PMA-induced shedding is not a direct consequence of protein kinase C activity, but rather results from protein kinase C-induced Erk MAP kinase signaling. This raises the possibility that protein kinase C may play a role in the activation of Erk MAP kinase signaling by growth factor receptors. However, we found that inhibition of protein kinase C signaling by chemical compounds, dominant-negative protein kinase C mutants or prolonged pre-treatment by PMA did not affect growth factor-induced shedding (data not shown). This suggests that protein kinase C does not play a role in growth factor-induced shedding through the Erk MAP kinase pathway.

The ability of Erk MAP kinase and the p38 MAP kinase signaling to induce ectodomain shedding in response to diverse stimuli strongly suggests that these pathways regulate shedding in a large variety of contexts. p38 MAP kinase signaling can be activated in response to various environmental stimuli, including osmotic shock, UV irradiation and inflammatory mediators, whereas Erk MAP kinase signaling is induced by growth factors, various tyrosine kinases and oncogenes (van der Geer *et al*., 1994; Seger and Krebs, 1995; Denhardt, 1996; Cohen, 1997). Therefore, a large variety of stimuli and changes in the cellular environment may lead to ectodomain shedding through the same signaling pathways. In addition, the activation of oncogenes, the increased growth factor stimulation and the up-regulation of MAP kinase signaling in tumor cells may be at the basis of increased shedding in tumor cells, which again may be controlled by these two MAP kinase pathways. Finally, the induction of ectodomain shedding of very diverse transmembrane proteins by these two MAP kinase pathways emphasizes the general nature of the control of ectodomain shedding. This is consistent with the notion that only a small family of metalloproteases may mediate ectodomain shedding for the many different transmembrane proteins. Considering the nature of the p38 and Erk MAP kinase pathways, it is tempting to speculate that a proximal phosphorylation event will play a key role in the activation of the protease(s) in response to the very diverse extracellular signals.

Materials and methods

Plasmids and reagents

pRK7α, an expression plasmid for transmembrane TGF-α, has been described previously (Shum *et al*., 1996). The L-selectin expression plasmid Leu8-14 (Migaki *et al*., 1995) was provided by Dr T.K.Kishimoto (Boehringer Ingelheim Pharmaceuticals, Inc.). The plasmid FGFR1′, encoding a cytoplasmically truncated FGFR1 (Wang *et al*., 1996), was provided by Dr C.W.Turck (University of California, San Francisco). Expression plasmids for wild-type MEK1, constitutively activated MEK1 (ΔN-S218E-S222D) and dominant-negative MEK1 (K97M) (Mansour *et al*., 1994) were provided by Dr N.G.Ahn (University of Colorado). A dominant-negative MEK2 expression plasmid (K101A MEK2) (Abbott and Holt, 1999) was provided by Dr J.T.Holt (Vanderbilt University), and the expression plasmid for constitutively activated Erk2 (MCMV5-Erk2-MEK1 LA) (Robinson *et al*., 1998) was provided by Dr M.H.Cobb (University of Texas Southwestern Medical Center). Plasmids encoding MKK6 (DD) and MKK6 (EE), two constitutively activated forms of MKK6, were provided by Dr M.Karin (University of California, San Diego). A plasmid that is essentially identical to MKK6 (DD), but was constructed independently, has been described (Raingeaud *et al*., 1996). TNF-α and cytoplasmically truncated PDGFRβ expression plasmids were constructed into the pRK vector using PCR-based approaches.

Acidic FGF and PDGF-BB were purchased from Calbiochem. Protein tyrosine kinase inhibitors SU5402 and AG1296 were obtained from Sugen and Calbiochem, respectively, and were used at 20 μM. Emetine and cycloheximide were purchased from Sigma, and used at 1 μg/ml. U0126 was obtained from Dupont and used at 5 μM. U73122, LY294002, herbimycin A and SB202190 were purchased from Calbiochem, and used at 10, 20, 20 and 10 μM, respectively.

Assay of transmembrane TGF-α cleavage

Fifty percent confluent CHO cells, cultured in F12 medium in 6-well plates, were transfected with the appropriate expression plasmids, as needed, using LipofectAmine reagent (Gibco-BRL). After 5 h incubation with Opti-MEM (Gibco-BRL), containing the mix of DNA and LipofectAmine reagent, cells were switched into F12 medium, containing 10% fetal bovine serum (FBS), and incubated for another 5 h. Cells were then cultured overnight in serum-free F12 medium. To analyze the levels of cell-associated TGF-α, cells were pulse-labeled with [^{35}S]methionine/cysteine in F12 medium for 20 min; free label was then removed by rinsing with cold medium and cells were allowed to incubate at 37°C in regular F12 medium/Dulbecco's modified Eagle's medium (DMEM) (1:1) containing 0.1% bovine serum albumin (BSA). FGF, PDGF or EGF (final concentrations 100 ng/ml), heat-inactivated FBS (20%) or PMA (10 nM) were added to the chase medium when necessary. At specified time points after initiation of the chase, cells were lysed in Tris-buffered saline (TBS) containing 1% NP-40. Transmembrane TGF-α in the cell extract was immunoprecipitated using the anti-TGF-α monoclonal antibody, α1mAb (Bringman *et al*., 1987; Shum *et al*., 1994), and the ^{35}S-labeled TGF-α forms were separated by SDS–PAGE and visualized by autoradiography (Shum *et al*., 1994).

To measure the levels of soluble TGF-α in the medium, [^{35}S]methionine/cysteine labeling was extended to 2 h. Cells were then rinsed three times with cold medium and incubated for 20 min, unless indicated otherwise, at 37°C in medium containing BSA with or without growth factors, serum or PMA. Medium was collected and soluble TGF-α was immunoprecipitated using the α1mAb (Shum *et al*., 1994). TGF-α was eluted from affinity beads by heating the samples in TBS containing 1% SDS, 10 mM dithiothreitol. Supernatants containing ^{35}S-labeled TGF-α were analyzed by SDS–PAGE analysis followed by autoradiography, or quantitated by liquid scintillation counting. To assess the effects of constitutively activated kinases on the basal level cleavage, the medium from transfected cells was collected and used for immunoprecipitation of TGF-α without a chase period. The d.p.m. for soluble TGF-α were normalized for transfection efficiency against the amount of transmembrane TGF-α immunoprecipitated from extracts of transfected cells that were labeled for 10 min, during which no cleavage would have occurred.

Assays for TNF-α or L-selectin cleavage in CHO cells

These assays were essentially the same as for TGF-α cleavage. ^{35}S-labeled transmembrane TNF-α extracted with TBS, 1% NP-40, or soluble TNF-α released into chase medium was immunoprecipitated with an anti-TNF-α polyclonal antibody (Santa Cruz Biotechnology) at 20 min after initiation of the chase, and analyzed by SDS–PAGE and autoradiography. Cleavage of ^{35}S-labeled L-selectin was allowed to take place for 10 min. Cell extracts were prepared in TBS, 1% NP-40, and full-length transmembrane L-selectin and the N-terminal fragment generated by ectodomain cleavage were immunoprecipitated with monoclonal antibody CA21 specific for the cytoplasmic domain of L-selectin (Kahn *et al*., 1994), separated by SDS–PAGE and visualized by autoradiography. Two forms (50 and 74 kDa) of transmembrane L-selectin were precipitated. Only the 74 kDa form is sensitive to ectodomain shedding, as previously observed

(Kahn *et al.*, 1994; Arribas *et al*., 1996). Soluble L-selectin released into medium was precipitated with monoclonal antibody DREG-56 (Kahn *et al.*, 1994), separated by SDS–PAGE and visualized by autoradiography.

Assay of neutrophil L-selectin shedding

Human neutrophils were isolated from EDTA-anticoagulated venous blood from normal healthy donors by dextran sedimentation and Percoll density gradient centrifugation (Dooley *et al*., 1982). After three washes with phosphate-buffered saline (PBS), neutrophils were resuspended at 5×10^6/ml in F12 medium supplemented with 25 mM HEPES. A total of 5×10^5 neutrophils were incubated for 15 min at room temperature in the presence or absence of U0126 and SB202190. Stimulation with fMLP (final concentration 100 nM) was performed at room temperature for 5 min and terminated by chilling the samples on ice. Cell surface L-selectin was stained with mouse monoclonal antibody DREG-56 (Feehan *et al*., 1996) at 10 μg/ml, followed by fluorescein-conjugated goat anti-mouse IgG. Incubations with the antibodies were performed on ice in PBS containing 1% BSA and 0.1% NaN_3. Immediately after staining, samples were analyzed by flow cytometry on a FACScan machine (Becton Dickinson, Mountain View, CA). For each treatment, 10 000 cells were analyzed.

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