The Elastin Receptor Complex Transduces Signals through the Catalytic Activity of Its Neu-1 Subunit*

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The binding of elastin peptides on the elastin receptor complex leads to the formation of intracellular signals but how this is achieved remains totally unknown. Using pharmacological inhibitors of the enzymatic activities of its subunits, we show here that the elastin-peptide-driven ERK1/2 activation and subsequent pro-MMP-1 production, observed in skin fibroblasts when they are cultured in the presence of these peptides, rely on a membrane-bound sialidase activity. As lactose blocked this effect, the elastin receptor sialidase subunit, Neu-1, seemed to be involved. The use of a catalytically inactive form of Neu-1 and the small interfering RNA-mediated decrease of Neu-1 expression strongly support this view. Finally, we report that N-acetyl neuraminic acid can reproduce the effects of elastin peptides on both ERK1/2 activation and pro-MMP-1 production. Altogether, our results indicate that the enzymatic activity of the Neu-1 subunit of the elastin receptor complex is responsible for its signal transduction, presumably through sialic acid generation from undetermined substrates.

Elastin is the extracellular matrix protein responsible for the elasticity of tissues. It is more abundant in tissues where resilience is required, such as skin, lung, ligaments, or large arteries (1). Elastin is constituted of tropoelastin molecules covalently bound to each other by covalent cross-links (2) and its hydrophobic and highly cross-linked nature make of it a very durable polymer experiencing essentially no turnover in healthy tissues (3).

The biological role of elastin was originally thought to be restricted to this mechanical function. However, when Senior et al. (4) demonstrated that elastin digestion products were chemotactic for neutrophils and macrophages, it became suddenly apparent that peptides derived from amorphous elastin could modulate cell physiology. In fact, it has been shown because fibroblasts (5–12), smooth muscle cells (7, 13–15), endothelial cells (16–19), leukocytes (20, 21), and lymphocytes (22) were sensitive to the presence of these peptides yielding a broad range of biological activities (see Ref. 23 for a review). A corollary of these observations was that those cells do express a receptor for elastin peptides.

The elastin receptor complex is constituted of three subunits, one peripheral 67-kDa subunit, which actually binds elastin, and two membrane-associated proteins of 61 and 55 kDa, respectively (10). The 67-kDa elastin-binding protein (EBP)2 binds the VGVAPG elastin sequence with high affinity. Additionally, EBP can be eluted from elastin affinity column by galactosugars suggesting that the elastin-EBP interaction could be regulated by galactosugars bound on a lectin site on EBP (10, 24). Consequently, galactosugars such as lactose are commonly used antagonists of EBP.

Later, the nature of this subunit was revealed by the work of Privitera et al. (25) who have shown that EBP is an enzymatically spliced variant of lysosomal β-galactosidase (β-Gal, EC 3.2.1.23). Consequently, it was hypothesized that the 61 and 55 kDa membrane-bound subunits corresponded to the lysosomal companions of β-Gal, respectively neuraminidase-1 (Neu-1, EC 3.2.1.18) and cathepsin A/protective protein (PPCA, EC 3.4.16.1) (26). This view was strongly supported by the fact that Neu-1 and PPCA are found at the plasma membrane of several cell types (27) and by the finding that β-Gal-related disorders are associated with elastic fibers abnormalities (28, 29). Finally, a very recent report has readily identified the elastin receptor complex as the association of these three subunits (30). In the lysosome, PPCA protects β-Gal and Neu-1 from intralysosomal digestion independently of its serine protease activity by forming a complex with these enzymes (26).

Neu-1 is a member of the sialidase family and catalyzes the hydrolysis of terminal sialic acid residues of oligosaccharidies, glycoproteins, and glycolipids (31, 32). Importantly, it has been shown in vitro that the removal of β-Gal or PPCA hinders the formation of active neuraminidase, indicating that all three

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2 The abbreviations used are: EBP, elastin-binding protein (accession number P16279); PMSF, phenylmethylsulfonyl fluoride; β-Gal, lysosomal β-galactosidase (accession number P16278); PPCA, protective protein/cathepsin A (accession number P10619); Neu-1, lysosomal neuraminidase (accession number Q99519); Neu5Ac, N-acetyl neuraminic acid; Neu5Ga2c, N-acetyl-α-D-neuraminic acid; Neu5Gc2c, N-glycolyl neuraminic acid; di Neu5Ac, 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid; pro-MMP-1, pro-collagenase-1; ERK, extracellular signal-regulated kinase; kE, k-elastin; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline.
components of the complex are required for neuraminidase activity (33).

Aside from its role in elastin assembly (30), several studies have pointed out that the elastin complex receptor is able to transduce intracellular signals (23). Our group has previously demonstrated that elastin hydrolysates and κ-elastin (kE), elastin peptides derived from the alcaline degradation of insoluble elastin, both stimulate the production of pro-MMP-1 in human skin fibroblasts (5) by activation of the ERK1/2 pathway (34, 35).

The induction of signaling pathways by the elastin receptor complex is now commonly accepted but remains discussed, notably because a critical issue is undocumented: how is the elastin peptides binding information processed by the receptor, i.e., how does the receptor transduce this signal from the extracellular face of the membrane to the cytoplasmic one? This work aims at answering this important question.

We show here that binding of elastin peptides to EBP stimulates Neu-1 sialidase activity. Using pharmacological and genetic techniques, we demonstrate that the induction of this sialidase activity is responsible for elastin receptor-dependent signaling assessed by measuring pro-MMP-1 production and ERK1/2 activation. Additionally, we show that the serum protease activity of PPCA is not required for this signaling. Finally, we suggest that the sialic acid released by Neu-1 activity could act as a second messenger and transduce elastin complex receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Elastin peptides were prepared as described previously (5). Briefly, insoluble elastin was prepared from bovine *ligamentum nuchae* by hot alkali treatment. Its purity was assessed by comparing its amino acid composition to that predicted from the elastin gene product. Soluble elastin peptides were obtained from insoluble elastin by organo-alkaline hydrolysis. This was achieved using 1 M KOH in 80% aqueous ethanol (36). The obtained mixture of elastin peptides is termed κ-elastin (kE) and exhibits the same biological and physical properties as elastin hydrolysates (5).

Lactose, aprotinin, phenylmethylsulfonyl fluoride (PMSF), 4-methylumbelliferyl-β-D-neuraminic acid, N-acetyl-α-D-neuraminic acid, 2,3-dehydro-2-deoxy-N-acetylenuraminic acid (ddNeu5Ac), proteases inhibitors mixture (reference P8340) and mouse monoclonal anti-β-actin antibody were from Sigma (Saint-Quentin Fallavier, France). Soluble elastin peptides were prepared as described previously (5). Briefly, elastin peptides were isolated from insoluble elastin by organo-alkaline hydrolysis. This was achieved using 1 M KOH in 80% aqueous ethanol (36). The obtained mixture of elastin peptides is termed κ-elastin (kE) and exhibits the same biological and physical properties as elastin hydrolysates (5).

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Silencer small interfering RNA (siRNA) and negative control siRNA were purchased from Ambion (distributed by Applied Biosystems, Courtaboeuf, France). Rabbit polyclonal phoso-specific antibodies against active forms of ERK1/2 (phosphorylated on Thr202 and Tyr204) were from Cell Signaling Technology Inc. (Beverly, MA, distributed by Ozyme, Saint Quentin en Yvelines, France). Rabbit polyclonal anti-Neu-1 antibody was purchased from Abcam (Cambridge, UK). The sheep anti-human MMP-1 polyclonal antibody was from Calbiochem (distributed by VWR International, Strasbourg, France). All reagents for cell culture and transfection reagent Lipofectamine 2000 were from Invitrogen (Cergy Pontoise, France). Enhanced chemiluminescence substrate kit was purchased from Amersham Biosciences Inc. (Orsay, France). N-Acetylenuraminic acid (Neu5Ac) was a generous gift of Dr. C. Augé (Laboratoire de Chimie Organique Multifonctionnelle, University of Paris-Sud, France). Others reagents were from Sigma.

**Expression Plasmids**—The −512ColA-luc plasmid encoding luciferase under the control of the MMP-1 promoter was generated by cloning the 5' upstream sequence of human collagenase-1 (MMP-1) into the pGL3Basic reporter plasmid (Promega, Lyon, France), as described previously (37). The plasmid encoding Neu-1 neuraminidase (pSCTop-Neu-1) was kindly provided by Dr. A. d’Azzo (Department of Genetics and Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis).

**Neu-1-G68V Construct**—The Neu-1-G68V mutant was obtained from the wild type plasmid using PCR site-directed mutagenesis. The target site was identified and two complementary oligonucleotides containing the desired mutation were synthesized (Invitrogen, Cergy-Pontoise, France). The sequence of the two oligonucleotides with mutated bases are 5’-CTG TGG TGT AGC ATG AGA CAG ATC GGC 3’ and 5’ GAC ACC CAC TCG CAC TCT GTC TAG CCG-3’. The reaction was performed in a final volume of 50 μl. The sample reaction contained 1 μl of Pfu Turbo DNA polymerase, 5 μl of 10X buffer (20 mM MgSO4), 50 ng of pSCTop-Neu-1 preparation, 125 ng of each oligonucleotide, 10 mM dNTP, and H2O. The reaction was performed using a Mastercycler gradient thermocycler (Eppendorf, Le Pecq, France) and subjected to the following program: denaturation of 30 s at 95 °C, 12 cycles of 30 s at 95 °C, 1 min at 55 °C and 6 min at 68 °C. The sample was digested by 1 μl of DpnI (10 unit/μl) for 3 h at 37 °C and chemically transformed into the XL1 Blue strain. Cells were cultured on a LB agar plate containing 50 μg/ml ampicillin and incubated overnight at 37 °C. The obtained clones were sequenced (IBMP, Strasbourg). The plasmid was then concentrated and purified by High Speed Maxi Prep kit (Qiagen, Courtaboeuf, France).

**Fibroblast Culture and Treatments**—Human skin fibroblast strains were established from explants of human adult skin biopsies obtained from informed healthy volunteers (aged 21–49 years). Cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/liter of glucose, glutamax I and pyruvate, supplemented with 10% fetal calf serum (FCS) and in the presence of 5% CO2. Cells at subcultures 4 to 8 were used. For experiments, fibroblasts were grown to confluence in medium containing 10% FCS. Before stimulation, cells were incubated for 18 h in DMEM supplemented with 0.5% FCS, washed twice with PBS, and incubated in serum-free DMEM with or without kE or Neu5Ac for the indicated times. The neuraminidase and serine protease inhibitors, 2,3-dehydro-2-deoxy-N-acetylenuraminic acid (ddNeu5Ac) and aprotinin or PMSF respectively, were incubated for 1 h before stimulation, whereas lactose (EBP antagonist) was incubated for 3 h. Inhibitors and antagonists were present in the cell culture media during the stimulation. Cell stimulation was stopped by adding ice-cold PBS containing 50 μM Na3VO4.
Signal Transduction by the Elastin Receptor

Measurement of Sialidase Activity at the Plasma Membrane of Fibroblasts—We used the protocol described by Lukong et al. (27) with minor modifications. The cells were seeded in 6-well culture dishes and washed several times with prewarmed PBS. They were then incubated in 1 ml of reaction buffer containing 20 mM CH₃COONa, pH 6.5, 0.4 mM 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid (Muf-NANA) with or without kE, in the absence or presence of ddNeu5Ac or lactose. After incubation, 400-μl aliquots of medium were added to 3.6 ml of 0.4 M glycine buffer (pH 10.4), and the fluorescent 4-methylumbelliferone product released in the medium was quantitated by exciting its fluorescence at 365 nm and recording its intensity at 445 nm.

Western Blotting—10⁶ fibroblasts or COS-7 cells were washed twice in ice-cold PBS containing 50 μM Na₃VO₄, scrapped, and sonicated in lysis buffer (PBS, pH 7.4, 0.5% Triton X-100, 80 μM glycerophosphate, 50 mM EGTA, 15 mM MgCl₂, 1 mM Na₃VO₄, and protease inhibitor mixture). Insoluble material was removed by centrifugation (20,000 × g, 20 min, 4°C). Protein concentrations were determined by bicinchoninic acid protein assay (Pierce; distributed by Interchim, Montluçon, France). Equal amounts of proteins were heated for 5 min at 100°C in Laemmli sample buffer, resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were placed in blocking buffer (5% (w/v) nonfat dry milk in Tris-buffered saline/Tween 20, 5% (w/v) nonfat dry milk in Tris-buffered saline/Tween 20) for 1 h at room temperature and incubated overnight at 4°C with anti-phospho-ERK1/2 (1:1000), anti-Neu-1 (1:500), or anti-β-actin (1:5000) antibodies. After five washings with Tris-buffered saline/Tween 20, the membranes were incubated for 1 h at room temperature in the presence of horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (1:4000 and 1:10,000 in blocking buffer, respectively).

For detection of pro-MMP-1, the culture media of fibroblasts were harvested and centrifuged (500 × g, 10 min, 4°C) to remove cellular debris. The supernatants were then concentrated using Microcon YM10 (Millipore, Molsheim, France) and equal amounts of proteins were subjected to SDS-PAGE and blotted as described above using the anti-human MMP-1 antibody (1:500) and a horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (1:4000 and 1:10,000 in blocking buffer, respectively).

Luciferase Assay of MMP-1 Promoter Activity—COS-7 cells were grown on 96-well plates to 80% of confluency (16,000 cells) in DMEM containing 4.5 g/liter of glucose, glutamax I, and pyruvate, supplemented with 10% FCS and in the presence of 5% CO₂. For transfection experiments they were incubated for 24 h in serum-free DMEM then with a mixture of Lipofectamine 2000 (5 μl) and Neu-1 or negative control siRNA (100 pmol). Cells were then washed twice with PBS then stimulated for the indicated time with kE. Cell stimulation was stopped by adding ice-cold PBS containing 50 μM Na₃VO₄ and ERK1/2 Western blots were conducted as described above.

RESULTS

EBP is required for elastin peptide signaling and it has been suggested that tropoelastin recruits EBP to the cell surface where the PPCA and Neu-1 reside thereby leading to the completion of the elastin receptor complex.

In the lysosome, Neu-1 activity is highly dependent on the full association of the β-Gal/PPCA/Neu-1 complex. Indeed, it has been shown that the association of PPCA to Neu-1 only results in a weak activity of the sialidase but that the addition of β-Gal to this assembly achieves its full activation.

By analogy to what is observed in the lysosome, we supposed that elastin peptide-driven recruitment of EBP to the membrane-bound PPCA/Neu-1 complex could stimulate the enzymatic activities of these sub-units and contribute to signal processing by the elastin receptor. As EBP is devoid of enzymatic activity, we analyzed the possible importance of PPCA and Neu-1 activities on elastin peptide signaling by the elastin receptor complex.

Inhibition of PPCA and Neu-1 Activities and Impact on Signaling by the Elastin Receptor Complex—In a previous work, we have shown that the treatment of human skin fibroblasts with elastin peptides resulted in the accumulation of pro-collagenase-1 (pro-MMP-1) in the culture medium (34), and that this effect was strictly mediated by ERK1/2 signaling (35). As a consequence, we have analyzed the influence of PPCA and Neu-1 inhibition on pro-MMP-1 accumulation and on ERK activation.

When cultured fibroblasts were treated with aprotinin, an inhibitor of serine protease, and further stimulated with kE, we observed that PPCA inhibition did not prevent pro-MMP-1 accumulation in the medium following kE treatment (Fig. 1A) nor ERK1/2 induction (Fig. 1B). These results were confirmed using PMSF (Figs. 1, C and D) which is an efficient PPCA inhibitor (39). As a consequence, we concluded that PPCA enzymatic activity was not required for proper signaling by the elastin complex receptor.
However, when the cells were treated with elastin peptides in the presence of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (ddNeu5Ac), a sialidase inhibitor (30, 40), pro-MMP-1 accumulation in the culture medium was reduced in a dose-dependent manner (Fig. 2A). As at 400 μM ddNeu5Ac, the effect of elastin peptides on pro-MMP-1 production was almost totally blocked, we decided to further use this experimental concentration. In the presence of ddNeu5Ac, elastin peptide-treated cells have lost their ability to activate ERK1/2 (Fig. 2A). As at 400 μM ddNeu5Ac, the effect of elastin peptides on pro-MMP-1 production was almost totally blocked. We decided to further use this experimental concentration. In the presence of ddNeu5Ac (10 μM), the effect of elastin peptides on pro-MMP-1 production was almost totally blocked. We decided to further use this experimental concentration. In the presence of ddNeu5Ac, elastin peptide-treated cells have lost their ability to activate ERK1/2 (Fig. 2A). As at 400 μM ddNeu5Ac, elastin peptide treatment promoted a sialidase activity at the surface of cells. Interestingly, elastin-driven activity could be totally blocked by 1 mM lactose suggesting that EBP signaling is involved in this phenomenon (Fig. 3, black circles). The use of ddNeu5Ac at the optimal concentration used to block elastin peptide-induced signaling resulted in a very low level of released fluorescence (Fig. 3, black squares) demonstrating that the membrane-residing sialidases were efficiently inhibited.

According to the literature, two distinct sialidase activities exist at the cellular surface. They correspond to the membrane targeted form of Neu-1 (27) and to peripheral Neu-3 (31, 41). Using the synthetic inhibitor ddNeu5Ac, we could not tell which of these two enzymes were actually involved in elastin peptide signaling. As a consequence, elastin peptide-induced signaling resulted in a very low level of released fluorescence (Fig. 3, black squares) demonstrating that the membrane-residing Neu-1 sialidase activity was required for elastin peptide signaling. However, we could not precisely determine which enzyme was involved as ddNeu5Ac is not specific of a sialidase form. As a consequence, we could not link this activity to that of the elastin receptor complex.

Analysis of Sialidase Activity at the Cell Surface of Fibroblast in Culture—The Muf-NANA compound is a neuraminidase substrate, which becomes fluorescent when it is cleaved by the enzyme. The product is detected at 445 nm when it is excited at 365 nm (27).

Using Muf-NANA, we have shown that untreated human skin fibroblasts exhibited a significant sialidase activity at their surface (Fig. 3, open circles). As a consequence the fluorescence level increased with time as the fluorescent product of Muf-NANA processing was released in the culture medium. When they were treated with kE (Fig. 3, open squares), this level was significantly raised demonstrating that elastin peptides treatment promoted a sialidase activity at the surface of cells. Interestingly, elastin-driven activity could be totally blocked by 1 mM lactose suggesting that EBP signaling is involved in this phenomenon (Fig. 3, black circles). The use of ddNeu5Ac at the optimal concentration used to block elastin peptide-induced signaling resulted in a very low level of released fluorescence (Fig. 3, black squares) demonstrating that the membrane-residing Neu-1 sialidase activity was efficiently inhibited.

Effects of Catalytically Inactive Neu-1 and Neu-1 siRNA Silencing on Elastin Receptor Complex Signaling—To determine the role of Neu-1 in the kE-induced signaling and MMP-1 expression, we generated a catalytically inactive form of Neu-1, Neu-1-G68V by site directed mutagenesis. This mutant form of Neu-1 exhibits no sialidase activity when transfected in COS-7
or human fibroblasts (42). Nevertheless, the G68V substitution seems to have little if no impact on the overall structure of the enzyme (43). Moreover, it has been shown that such a mutant is able to reach the lysosomes (44). As a consequence, we hypothesized that this mutant could be present not only in the lysosomal complex but also that it could be targeted to the cell membrane where it would compete with normal Neu-1.

Because of the difficulty in achieving an efficient co-transfection of plasmids expressing Neu-1-G68V and construct encoding luciferase under the control of MMP-1 promoter in human skin fibroblasts, we used COS-7 cells, a model, which was successfully used in previous Neu-1 and elastin-related experiments (42, 45).

When cells were transfected with the empty vector (Fig. 4A), kE treatment led to a 60% increase in luciferase activity. However, when COS-7 expressed Neu-1-G68V, the effect of elastin peptides on MMP-1 promoter activity was significantly reduced demonstrating the requirement of Neu-1 sialidase activity in elastin peptide signaling.

To confirm these results, we checked the influence of Neu-1 siRNA on kE-induced ERK1/2 phosphorylation. We observed that, 24h after cell transfection, the expression of Neu-1 was decreased by 83% in cells transfected with the Neu-1 siRNA, whereas it was unchanged in those transfected with the siRNA negative control (Fig. 4B). kE-driven ERK1/2 phosphorylation was nearly totally inhibited in Neu-1 siRNA-transfected cells while no effect was observed in negative control siRNA-transfected cells (Fig. 4C).

**Individual Role of Sialic Acid on Signaling by the Elastin Receptor Complex**—As our results obtained using either ddNeu5Ac or Neu-1-G68V or Neu-1 siRNA suggested that elastin peptide-induced signaling occurred via Neu-1 activity, we analyzed the influence of Neu5Ac on cells, monitoring both ERK1/2 activation and pro-MMP-1 production (Fig. 5). We observed that ERK1/2 activation in fibroblasts was sensitive to sialic acid in a dose-dependent manner (Fig. 5A). Thus sialic acid could reproduce the effects of elastin peptides.

As the addition of an acidic compound to the culture medium could have changed its pH, the pH of the media were measured before and after acid addition (data not shown) but the pH was unchanged thereby excluding any possible unwanted stress caused by acid addition. Moreover, we showed that fibroblasts stimulation with Neu5Ac led to pro-MMP-1 production (Fig. 5B).

Finally, we stimulated fibroblasts with sialic acid when Neu-1 activity was blocked by addition of ddNeu5Ac in the culture medium to confirm that Neu5Ac-induced signaling was related to its actual signaling capacity. We evidenced that Neu5Ac could induce ERK1/2 phosphorylation independently of Neu-1 activity (Fig. 5C). As a consequence, our results indicated that Neu5Ac alone could reproduce the effect of elastin peptides on fibroblasts.

**DISCUSSION**

Elastin peptides modulate several biological processes such as cell proliferation, chemotaxis or proteases release (23). The corollary between these effects seems to be the ability of elastin peptides to induce intracellular signaling by activation of the elastin receptor complex. Although the signaling pathways induced by elastin peptides have been studied in some cases, the operational mechanism of their cognate receptor remained totally unknown. Our work strongly suggests that the elastin complex receptor operates through an original mechanism during which the signal of elastin peptides binding on the EBP sub-unit is processed by activation of the catalytic activity of Neu-1 and subsequent sialic acid generation.

Elastin peptides binding to EBP is essential for the induction of signaling pathways as demonstrated by the use of EBP antagonists (13, 35, 46). As a consequence, EBP is sometimes reported as being the elastin receptor. Moreover, the role of the two other subunits and their respective functions in the elastin receptor complex signaling events had never been studied and especially the putative requirement of their enzymatic activities.

To check the involvement of PPCA and Neu-1 in elastin peptide signaling, we assessed ERK1/2 activation as it was demonstrated that elastin peptides could trigger this pathway in several cell types (13, 35, 47). Additionally, we analyzed pro-MMP-1 accumulation in the culture media because this is an ERK1/2-governed phenomenon in human skin fibroblasts. We show here that the abrogation of serine-protease activities at the cell membrane has no effect neither on pro-MMP-1 production nor ERK1/2 activation (Fig. 1) but that blocking of membrane-bound sialidase activity using the sialidase inhibitor ddNeu5Ac (30) totally blocks these elastin peptide-regulated events (Fig. 2). Our data consequently suggest that a serine protease activity does not participate in elastin peptide-induced signaling, thereby excluding the possibility that PPCA catalytic activity is involved in the signal transduction by the elastin...
receptor complex. However, they also pointed out that a sialidase activity was required for elastin complex receptor signaling but, at that point, we could not tell if it was a membrane or cytosolic enzyme. Consequently, we analyzed the influence of elastin peptides fibroblasts stimulation on plasma membrane sialidase activity and found that cell stimulation with elastin peptides drastically increased global sialidase activity at the plasma membrane of cultured cells (Fig. 3). Importantly, the use of an EBP antagonist, at a concentration which totally blocks EBP-induced signaling in fibroblasts (35), inhibited elastin peptide-induced sialidase activation. We stress here that the observed sialidase activity following cell stimulation could not be attributed to parasite measurement of lysosomal sialidase but only to membrane-bound sialidase. Indeed, it has been clearly established that the optimum pH for the lysosomal enzyme is acid (pH 4.5) unlike the plasma membrane bound sialidase activity which has an optimum pH of 6.5 (31). Moreover, EBP is never targeted to the lysosome (25, 48, 49) excluding the possibility that elastin peptides could activate lysosomal sialidase via EBP. Altogether, these observations prompted us to conclude that elastin peptides treatment induced a sialidase activation at the plasma membrane. This finding suggested that Neu-1, the sialidase of the elastin receptor complex, could be involved.

Two sialidases are found at the plasma membrane of cells and originate from Neu-1 and Neu-3 isoforms (27, 31, 41). As it had been shown very recently that Neu-1 was the isoform found in the elastin complex receptor (30, 50), we evaluated its involvement in elastin signaling by generating an enzymatic inactive form of Neu-1, Neu-1-G68V, which exhibits no sialidase activity (42). Due to the difficulty in achieving a convenient transfection rate using primary human dermal fibroblasts, we used COS-7 cells, a model previously used in elastin- and Neu-1-related experiments (42, 45).

We show that COS-7 cells are sensitive to elastin peptides stimulation as demonstrated by MMP-1 promoter activity analysis (Fig. 4A). As expected, transfection of cells with a construct encoding Neu-1-G68V significantly reduced kE-induced luciferase expression placed under the control of MMP-1 promoter reinforcing our former hypothesis. In order to confirm the role of Neu-1 in elastin peptide-induced signaling, we performed siRNA experiments to reduce Neu-1 expression (Fig. 4B) and checked its influence on ERK1/2 activation. We demonstrate that siRNA-driven Neu-1 knockdown led to an inhibition of kE-induced ERK1/2 activation (Fig. 4C). Altogether, our results demonstrated that Neu-1 sialidase activity is
FIGURE 5. Fibroblast stimulation with Neu5Ac reproduces kE effect on ERK1/2 activation and pro-MMP-1 production. A, Western blots analysis of cellular extracts. Cells were stimulated for 30 min with various doses of Neu5Ac or with kE (50 µg/ml). Membranes were probed with anti-phospho-Thr202/Tyr204-ERK1/2 polyclonal antibodies. To demonstrate equal loading, blots were stripped and reprobed with an anti-β-actin antibody. Blots are representative of three independent experiments with similar results. B, Western blots analysis of pro-MMP-1 production. Cells were stimulated or not with Neu5Ac (10 µM) for 24 h. The culture media were then harvested and assessed for pro-MMP-1 presence. Only the nonglycosylated (53 kDa) form of pro-MMP-1 is detected (see Fig. 1 legend). Blots are representative of three independent experiments with similar results. C, Western blots analysis of cellular extracts. Cells were stimulated for 30 min with Neu5Ac (10 µM) in the presence or absence of ddNeu5Ac (400 µM). Membranes were probed with anti-phospho-Thr202/Tyr204-ERK1/2 polyclonal antibodies. To demonstrate equal loading, blots were stripped and reprobed with an anti-β-actin antibody. Blots are representative of three independent experiments with similar results.

triggered through an EBP-dependent mechanism and is necessary for elastin peptide-signaling induction.

Previous works have suggested that sialidase activity of the receptor could participate in elastin assembly via desialylation of glycoproteins of the microfibrillar network (31) and this hypothesis has been confirmed very recently (30). In this model, tropoelastin is bound to EBP which, when it is secreted, binds to the rest of the complex allowing removal of terminal sialic acids from glycoproteins by Neu-1. Consequently, the unmasked galactosyl moieties can interact with the galactolectin domain of EBP, leading to the local release of tropoelastin. This model implicates that sialidase activity is regulated by the binding of EBP. This point correlates with our results. Additionally, the stabilizing effect of EBP on sialidase activation perfectly match that of β-Gal in the lysosomal complex. Indeed, it has been shown that association of β-Gal to the rest of the complex is an absolute requirement for the generation of a fully active Neu-1 (33). Consequently, it can be considered that EBP behaves as a “switch” molecule, which detects the agonists and transfers this information (by an undescribed mechanism) to the transducing subunit of the receptor, i.e. Neu-1. The precise role of PPCA in the complex remains undefined but it seems reasonable to suggest that it could contribute to the stabilization of the receptor architecture.

The role of sialidase activity in signal transduction has already been reported. Indeed, it has been shown that anti-CD3e-induced IL-4 production by splenocytes required neuraminidase activity and that incubation of these cells using bacterial neuraminidase alone induced IL-4 expression (52). Moreover, dermal fibroblasts or monocytes incubation with bacterial neuraminidase respectively led to cell proliferation and ERK1/2 activation through desialylation of unidentified plasma membrane glycoconjugates (53, 54). These data support our proposal that Neu-1 activity could contribute to elastin peptide signaling.

Neu-1 catalyzes the removal of terminal sialic acid residues from oligosaccharides, glycoproteins, and glycolipids (31, 32). The consequence is the release of sialic acid in the cellular environment. Sialic acids represent a family of about 40 derivatives of the nine-carbon sugar neuraminic acid. The most widespread form of sialic acid is Neu5Ac and sialidases are specific for Neu5Ac linked to the subterminal sugar chain of diverse poly- and oligosaccharides glycoconjugates (55). As a consequence, we evaluated the ability of Neu5Ac to reproduce the effect of elastin peptides on ERK1/2 activation. Our results (Fig. 5A) show that Neu5Ac treatment leads to an activation of ERK1/2 in a dose-dependent manner, thereby reproducing the effect of kE on this pathway. We also show that addition of Neu5Ac to the culture medium prior to cell stimulation does not modify its pH thereby eliminating the possibility of parasite ERK1/2 activation due to unwanted pH variation. Moreover, the stimulation of fibroblasts with Neu5Ac led to pro-MMP-1 accumulation in the culture medium (Fig. 5B). Finally, we demonstrated that Neu5Ac stimulated ERK1/2 phosphorylation even when Neu-1 activity was blocked by ddNeu5Ac (Fig. 5C), demonstrating its actual signaling capacity. These data strongly suggest that sialic acid release following Neu-1 activation could be responsible for elastin peptide-induced ERK1/2 activation.

It is, to our knowledge, the first time that a role of signaling messenger is directly attributed to a member of the sialic acid family in its free form. Indeed, regulation of signaling events by sialic acid has already been described but its role was that of a mediator in molecular interactions during signaling. For instance, Neu5Ac from GM3 ganglioside glycan moiety is essential for the ability of this glycosphingolipid to interact with the EGF receptor leading to the inhibition of its kinase activity (56). Moreover, it has been demonstrated that CD22 interacts with a sialic acid residue of BCR leading to an inhibition of its signaling (57, 58). Nevertheless, to our knowledge, a role of signaling inducer has not been reported for Neu5Ac. More work is now necessary to identify the substrate(s) of Neu-1 and disclose the mechanisms by which Neu5Ac activates ERK1/2 pathway. Experiments are now underway to answer these questions.

In conclusion, this work constitutes the first proposal for the mechanism by which the elastin receptor complex transduces its signals. It brings important data concerning the operational mechanism of the receptor and strongly suggests that this proc-
ess involves EBP-dependent Neu-1 activation leading to sialic acid cleavage from unknown substrates. In our proposal, sialic acid could be the second messenger responsible for elastin peptide-induced signaling.

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