Chapter 5

Results

Although it is known that thrombin–induced contractile protein expression and the subsequently VSM cell differentiation requires a biphasic ERK1/2 activation, the molecular mechanism which leads to the first and the long–lasting ERK1/2 phosphorylation are poorly understood. The aim of this thesis was to elucidate, the molecular basis of the thrombin–induced ERK1/2 phosphorylation.

5.1 Second phase signaling through the Ras/Raf/MEK/-ERK pathway in thrombin–stimulated VSM cells requires *de novo* RNA and protein synthesis

Previous studies have shown that the thrombin–induced contractile protein expression in VSM cells requires a biphasic activation of members of the Ras/Raf/MEK/ERK signaling cascade. To analyze whether both phases of activation depend on the phosphorylation of preexisting signaling molecules or whether *de novo* mRNA transcription and translation into a protein may be required, the effect of inhibiting *de novo* RNA transcription or protein synthesis on the second phase of MEK and ERK1/2 activation by thrombin stimulation

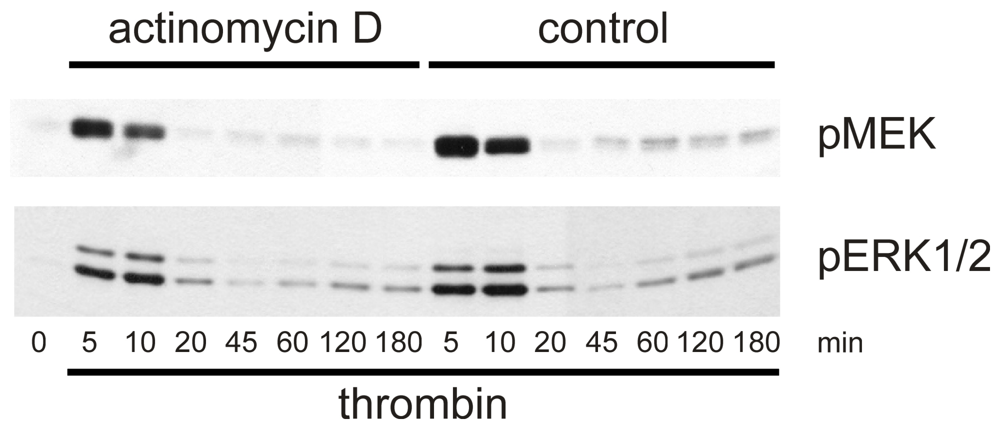

Figure 5.1: Requirement of *de novo* RNA transcription for the second phase of MEK and ERK1/2 activation by thrombin. Primary cultures of newborn rat aortic VSM cells were kept in serum–free medium for 36 h, treated with actinomycin D (4 μM) to block RNA transcription, or maintained in serum–free medium (control). Cells were then stimulated with thrombin (2 U/ml) for up to 180 min and lysed. Proteins were separated on 10% SDS–PAGE, electroblotted, and probed with anti–phospho MEK or ERK1/2 antibodies. Shown are blots on which samples with and without the respective inhibitor were processed in parallel.

was analyzed.

Actinomycin D is a well known cyclic polypeptide–containing antibiotic that binds to DNA and inhibits the RNA synthesis by blocking the progression of mRNA polymerase. VSM cells were seeded in 35–mm culture dishes, maintained in serum–free medium for 36 h, and pretreated with 4 μM actinomycin D 30 min prior to thrombin stimulation (2 U/ml). Non–pretreated VSM cells were used as a control. The detection of the protein kinases was carried out by rabbit polyclonal anti–phospho MEK, and anti–phospho ERK1/2 antibodies. MEK was expressed in three different isoforms: MEK–1a (45 kDa), MEK–1b (41 kDa) and MEK–2 (46 kDa) [192], whereas ERK was expressed in two isoforms: ERK1 (44 kDa) and ERK2 (42 kDa) [193]. As shown in figure 5.1, thrombin–induced second phase of MEK activation is completely abolished by actinomycin treatment, whereas the early phase of thrombin–stimulated phosphorylation remained unaffected. The results obtained when the blots were probed with anti–phospho ERK1/2 antibody contrasted with the MEK activity. Actinomycin D treatment (4 μM added 30 min prior to thrombin stimulation) inhibited approximately 50% of the thrombin–induced second phase of ERK1/2 activation, whereas

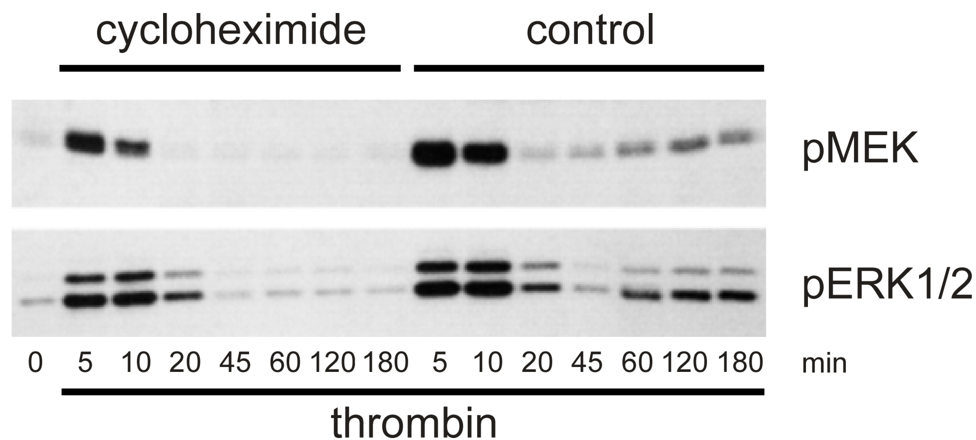

Figure 5.2: Requirement of protein synthesis for the second phase of ERK1/2 activation by thrombin. Serum starved VSM cells were treated with 10 μM cycloheximide for 30 min to block the protein synthesis and then stimulated with thrombin (2 U/ml) for up to 180 min and lysed. Proteins were separated on 10% SDS–PAGE, electroblotted, and probed with anti–phospho MEK or ERK1/2 antibodies. Blots where samples with and without the respective inhibitor were processed in parallel are shown.

the long–lasting MEK activation was completely blocked by inhibitor treatment.

To probe if the second phase of the ERK1/2 phosphorylation by thrombin stimulation requires not only *de novo* RNA transcription but also *de novo* protein biosynthesis, VSM cells were treated with cycloheximide. Cycloheximide is an inhibitor of the protein biosynthesis by interfering the mRNA translocation. Serum starved cells were pretreated with 10 μM cycloheximide for 30 min and stimulated with 2 U/ml thrombin. As before, non–pretreated VSM cells served als contol. As shown in figure 5.2, the thrombin–induced long–lasting MEK and ERK1/2 activation was completely inhibited by blocking the mRNA translocation and protein biosynthesis. Both results suggest that stimulation of VSM cells with thrombin activates a mechanism that leads to *de novo* RNA synthesis and protein expression. This thrombin–induced gene expression and protein synthesis is necessary for triggering a second, long–lived phosphorylation of MEK and ERK1/2, which is associated with the expression of contractile proteins in of VSM cells.

5.2 Delayed ERK1/2 phosphorylation is linked to vesicular transport

The necessity of *de novo* protein expression in response to thrombin stimulation in VSM cells, which correlates with the second phase of the ERK1/2 activation, was demonstrated in the previous section. To elucidate which proteins may play a key role in the thrombin–induced VSM cells redifferentiation, the transcription kinetic of different proteins was analyzed. The first objective was to examine the immediate–early gene expression. Immediate–early genes are activated transiently and rapidly in response to multiple stimuli and represent the first response mechanism that is activated at the transcriptional level in response to stimuli. An analysis of c–jun and c–fos transcription kinetics revealed that the expression of these transcripts is upregulated 5 min after thrombin stimulation and subsequently rapidly decreased.

Since the expression of the immediate–early genes did not temporally correlate with the second phase of the ERK1/2 phosphorylation, the key protein could be part of the second wave of gene expression. In figure 1.3 (Introduction), a hypothetical signaling pathway in response to thrombin stimulation is outlined. Multiple proteins with important roles in the transmission of the thrombin–induced ERK1/2 activation signal are indicated. To delineate candidate proteins that are essential for the thrombin induced second phase of ERK1/2 phosphorylation, we tested whether cytoskeletal components and trafficking processes are involved in signal transduction. After translation, plasma membrane–resident proteins are translocated into the ER where they are post-translationally modified. Plasma membrane–resident proteins and proteins determined for secretion must pass through the Golgi apparatus, and vesicles containing the proteins are transported to their final destination into the plasma membrane via cytoskeletal cargo routes. The knowledge about the influence of protein trafficking or cytoskeletal elements to the signaling of the thrombin–induced second phase of ERK1/2 activation could help narrow down the candidate proteins.

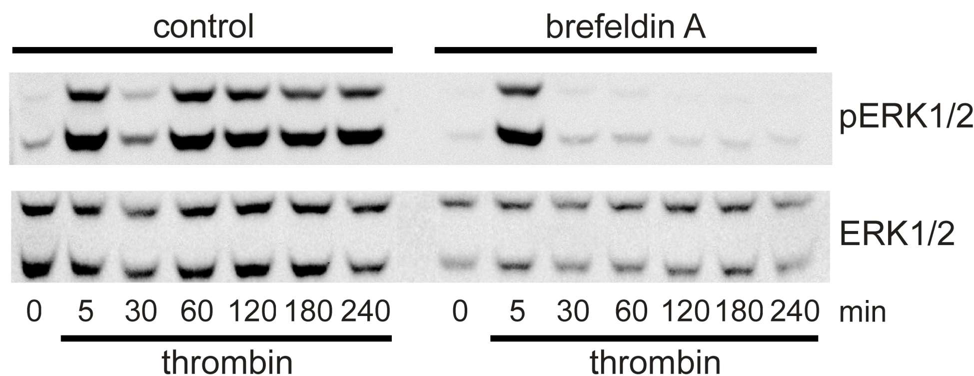

Figure 5.3: Effects of disruption of vesicular transport on the second phase of ERK1/2 activation in VSM cells. VSM cells were incubated for 36 h in serum–free medium, pretreated with 7 μM brefeldin A for 30 min, and then stimulated with 2 U/ml thrombin. Whole–cell lysates were separated on a 10% SDS–PAGE, electroblotted and probed with anti–phospho ERK1/2 and anti–ERK1/2. This antibody recognizes a dual phosphorylation at Thr202 and Tyr204 of ERK1 (Thr185 and Tyr187 of ERK2).

Inhibition of the late phase of ERK1/2 in response to thrombin by disruption of the vesicle transport could clarity if plasma membrane proteins are involved in the signaling of the long–lasting ERK1/2 activation.

To elucidate if the protein trafficking between the ER and the Golgi apparatus is required for the second phase of ERK1/2 activation in response to thrombin stimulation, we induced a disruption of protein transport applying brefeldin A. Brefeldin A is an inhibitor of Sec7–mediated guanine nucleotide exchange of Arf GTPases and thereby blocks the assembly and loading of cargo to vesicles in the exocytic ER–to–Golgi transport. VSM cells were grown in 35–mm culture dishes and maintained in serum–free medium for 36 h. Serum starved cells were pretreated with brefeldin A (7 μM) 30 min prior to thrombin stimulation (2 U/ml) and then stimulated for up to 240 min and lysed. Non–pretreated VSM cells were used as control. As shown in figure 5.3, thrombin stimulation of VSM cells induced a strong first and second phase of ERK1/2 activation. The inhibition of the vesicular trafficking between the ER and the Golgi apparatus by brefeldin A treatment completely abolished the second phase of ERK1/2 activation, whereas the first phase remained. The unphosphorylated forms of ERK1/2 served as loading controls and demon-

strated that equal amounts of proteins were utilized in the assay (lower panel). The result of the experiment indicate that intact transport between both structures is necessary for the signaling of the thrombin–induced second phase of ERK1/2 phosphorylation.

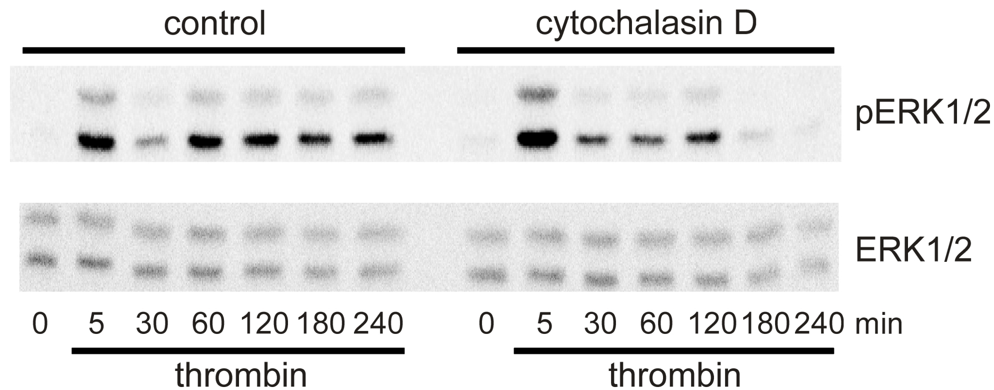

Figure 5.4: Effects of actin depolymerisation on the second phase of ERK1/2 activation in VSM cells. Cells were incubated for 36 h in serum–free medium, pretreated with 2 μM cytochalasin D for 30 min, and then stimulated with 2 U/ml thrombin. Whole–cell lysates were separated on a 10% SDS–PAGE, electroblotted and probed with anti–phospho ERK1/2 and anti–ERK1/2.

To test whether the second phase of ERK1/2 activation by thrombin stimulation is influenced by blocking microtubule–dependent vesicular transport, nocodazole was applied, which inhibits microtubule polymerization by binding to β–tubulin and, thus, preventing the formation of one of the two interchain disulfide bridges. VSM cells were pretreated with 1 μg/ml nocodazole for 30 min and then stimulated with thrombin for up 240 min. The analysis of the blots revealed that the second phase of ERK1/2 phosphorylation is unaffected by microtubule disruption (data not shown).

Since the last phase of the ERK1/2 activation in response to thrombin was not dependent on an intact tubulin cytoskeleton, the influence of disrupting actin–depending vesicle trafficking in VSM cells on ERK1/2 phosphorylation after thrombin stimulation was tested. VSM cells were pretreated with cytochalasin D, a fungal metabolite that binds to actin filaments and blocks the polymerization and the elongation of actin. Serum-starved VSM cells were pretreated with 2 μM cytochalasin D for 30 min and, subsequently, stimulated

with thrombin (2 U/ml) for up to 240 min.

As shown in figure 5.4, the first phase of the ERK1/2 activation by thrombin stimulation is unaffected by cytochalasin D treatment. However, the long–lasting ERK1/2 phosphorylation is reduced to approximately 50% 60 min after stimulation and is completely abolished after 180 min. Since actin depolymerisation may also affect the constitutive or agonist–triggered release of preformed secretory vesicles, possibly containing active matrix metalloproteinases that are important in the transactivation of the EGFR, brefeldin A may trigger more selective effects.

Inhibition of the thrombin–induced second phase of the ERK1/2 activation by brefeldin A and cytochalasin D indicated that the signaling pathway which leads to VSM cell redifferentiation, requires the synthesis and vesicle transport to the plasma membrane of a plasma membrane–resident or secretory protein.

5.3 Expression of HB–EGF is upregulated in response to thrombin treatment in VSM cells

Candidate genes that are expressed upon thrombin stimulation in VSM cells and that are required for a EGFR transactivation and second phase of ERK1/2 phosphorylation include the receptor proteins PAR1 and EGFR as well as pro–HB–EGF. These proteins are cleaved and/or internalized after stimulation and may, thus, require a constitutive or thrombin–induced replenishment. Additionally, the localization of the three proteins at the plasma membrane convert these proteins in perfect candidates. Additionally, HB–EGF expression is strictly modulated and several regulatory factors have been expounded, some of which indicate roles of HB–EGF in physiological and pathological processes [113], including atherosclerosis [136]. In addition, HB–EGF expression is upregulated in atherosclerotic plaques [136], indicating that the growth factor could be involved in the development of

this disease.

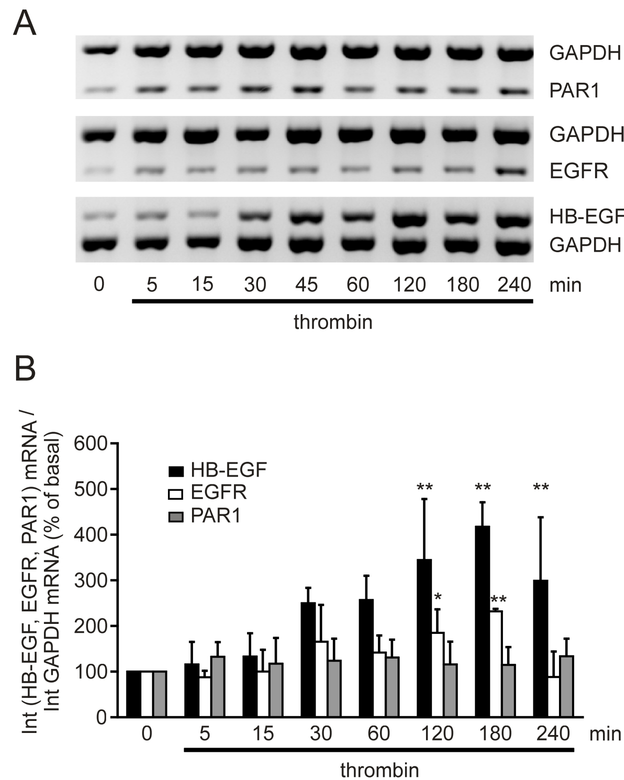

Figure 5.5: Thrombin–induced transcription of EGFR and HB–EGF. A, Serum–starved VSM cells were stimulated with 2 U/ml thrombin for up to 240 min. The transcription was analysed via multiplex RT–PCR of PAR1, EGFR and HB–EGF. PCR products were separated on 1.5% agarose gels, stained, and imaged with a CCD–camera. B, Quantitative analysis of results from 3 independent experiments performed as shown in (A). Statistical significance was tested using a Student´s unpaired t–test (comparison of band intensities with and without modulators) or a paired t–test (comparison of stimulated samples to basal signal intensities). Significance was accepted at $p < 0.05$ (*) or $p < 0.01$ (**).

VSM cells were seeded in 66–mm culture dishes, maintained in serum–free medium for 36 h, stimulated with 2 U/ml thrombin for up 240 min and harvested. RNA extraction and reverse transcription was performed and the gene expression of PAR1, of HB–EGF and of EGFR was tested semi–quantitatively by a multiplex PCR applying non–saturating numbers of amplification cycles and co–amplification of the constitutively expressed glyceraldehyde 3–phosphate dehydrogenase (GAPDH) gene transcript as an internal control. Quantification of fluorescence intensities and normalization to the transcription of GAPDH

revealed that the transcription of HB–EGF was increased about 3–fold (Fig. 5.5). The time course of HB–EGF transcription showed a first significant increase of the HB–EGF expression 30 min after thrombin stimulation and remained elevated for at least up 3 h. Since the expression of the immediate–early genes c–jun and c–fos was induced already 5–10 min after thrombin stimulation of VSM cells, expression of HB–EGF is not part of the first wave of gene expression events. In addition, analysis of EGFR transcription indicated an upregulation approximately 60 min after stimulation, which was maximal after 120 min. At this time point EGFR transcription was about 2–fold increased compared to non–stimulated cells. However, the transcription of PAR1 was unaffected by thrombin stimulation in primary VSM cells.

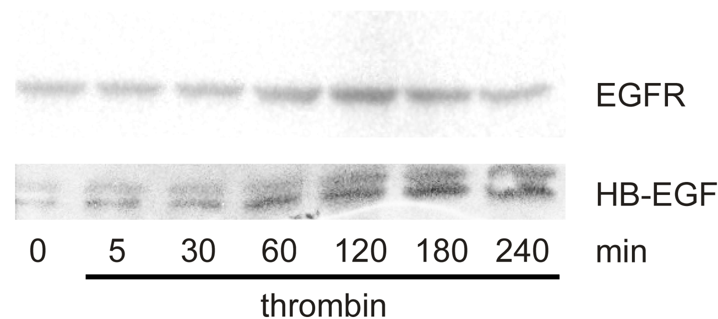

Figure 5.6: Thrombin–induced expression of EGFR and HB–EGF. Quiescent VSM cells were stimulated with 2 U/ml thrombin for the indicated times. Whole–cells lysates were subjected to 10% SDS–PAGE, electroblotted, and probed with anti–HB–EGF and anti–EGFR antibodies.

To confirm that the expression of HB–EGF and EGFR is upregulated by thrombin stimulation, an analysis of whole cell lysates was performed. Serum-starved VSM cells were stimulated with thrombin for up 240 min and then lysed. Western blot analysis of HB–EGF expression demonstrated at the protein level that the expression is upregulated 60 min after thrombin stimulation and exhibits a maximum after 180 min (Fig. 5.6). Analysis of the EGFR expression revealed an upregulation of the protein levels 60 min after stimulation with a maximal rate of expression 120–180 min after thrombin stimulation. HB–EGF and EGFR expression rates correlated with the transcription kinetics analysis of both proteins.

Thus, we conclude that thrombin–stimulated VSM cells exhibit an upregulation of HB–EGF transcription, which correlates with the second phase of the ERK1/2 activation. This results support the hypothesis that the signaling, which leads to the long–lasting ERK1/2 phosphorylation in response to thrombin, requires *de novo* expression of a plasma membrane protein.

5.4 Trafficking of HB–EGF

Analysis of thrombin–induced HB–EGF transcription kinetic and expression rates of the protein revealed that the transcription and expression of the protein are upregulated and coincided with the second phase of the ERK1/2 activation. Since *de novo* synthesized pro–HB–EGF has to be shed to yield soluble HB–EGF which activates the EGFR and the MAPK signaling pathway, the plasma membrane protein would have to be rapidly synthesized and processed after thrombin stimulation. To demonstrate that the newly

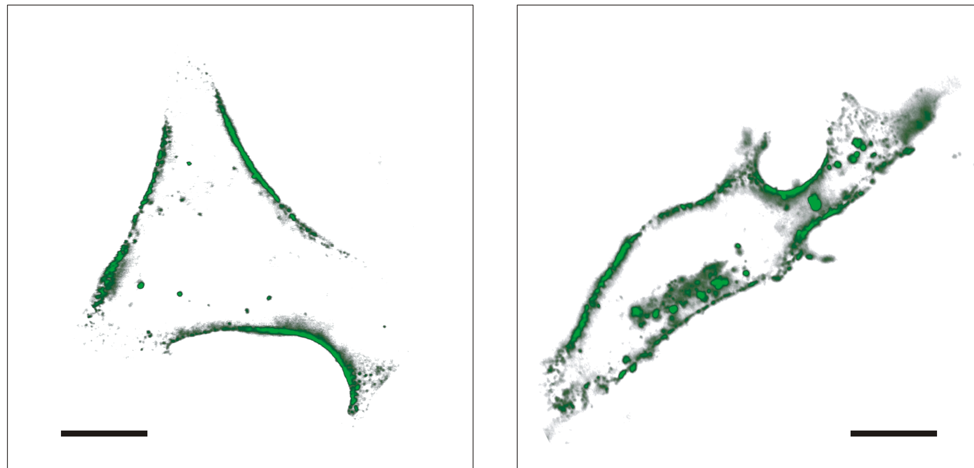

Figure 5.7: Plasma membrane trafficking of HB–EGF in VSM cells. VSM cells were nucleofected with an expression plasmid encoding a C–terminal YFP–fused HB–EGF construct and seeded on glass coverslips previously coated with poly–L–lysine. Five hours after nucleofection, cells were imaged by confocal laser scanning microscopy (Zeiss LSM–510META, equipped with an α–Plan Fluar 100x/1.45 objective). YFP was excited at 488 nm and emitted light was detected through a 505–nm long pass filter. Scale bar, 10 μm.

synthesized protein can localize to the plasma membrane in time with the activation of the EGFR and the subsequent second phase of ERK1/2 activation, a cDNA plasmid encoding a C–terminal YFP–tagged HB–EGF construct was designed. VSM cells were nucleofected with 5 μg of the cDNA plasmid encoding a HB–EGF–YFP construct and seeded on poly–L–lysine–coated glass coverslips. The localization of the fluorescent HB–EGF fusion protein was analyzed by confocal laser scanning microscopy. The HB–EGF–YFP protein was efficiently trafficked to the plasma membrane as early as 5 h after nucleofection (Fig. 5.7). This time was the earliest time point at which the fluorescent protein was reliably detectable (probably due to the need for an autooxidation–dependent maturation of the YFP fluorochrome). This result demonstrates that HB–EGF–YFP is rapidly processed and that the trafficking of the protein to the plasma membrane is a fast and efficient process.

5.5 HB–EGF trafficking is inhibited by brefeldin A and cytochalasin D treatment

As demonstrated before (see 5.3, 5.4), treatment with brefeldin A or cytochalasin D inhibits the thrombin–induced long–lasting ERK1/2 activation in VSM cells. Therefore, the effect of the protein transport disruption on the subcellular localization of HB–EGF in living cells was investigated in a next step. To this end, VSM cells were nucleofected with a cDNA plasmid encoding a C–terminally YFP–tagged HB–EGF construct, seeded on poly–L–lysine–coated glass coverslips and treated with the respective blockers 1 h after nucleofection. As before, the localization of the fluorescent HB–EGF fusion protein was analyzed by confocal laser scanning microscopy. Again, in untreated VSM cells, the HB–EGF–YFP protein was rapidly trafficked to the plasma membrane within 5 h after nucleofection (Fig. 5.8, A). However, treatment of the cells with inhibitors of protein transport resulted in an altered localization of HB–EGF–YFP. Inhibition of the secretory

pathway with brefeldin A (7 μM) resulted in a localization of HB–EGF–YFP in a reticular compartment including the nuclear membrane, presumably the ER (Fig. 5.8, B). It is known that brefeldin A blocks the transport between the ER and the Golgi apparatus. The disruption of the normal structure of the ER and the Golgi apparatus is also visible in figure 5.8, B. On the other hand, in VSM cells that were treated with cytochalasin D (2 μM), the HB–EGF–YFP fusion protein remained in intracellular vesicular structures (Fig. 5.8, C). It is worth mentioning, that experiments in transiently transfected HEK293 fibroblasts essentially confirmed these results, but demonstrated a less efficient integration of HB–EGF into the plasma membrane compared to that observed in VSM cells. Thus, the disruption of the second phase of ERK1/2 phosphorylation in thrombin–stimulated VSM cells by cytochalasin D or by brefeldin A may indeed reflect the inhibition of plasma membrane trafficking of newly synthesized HB–EGF.

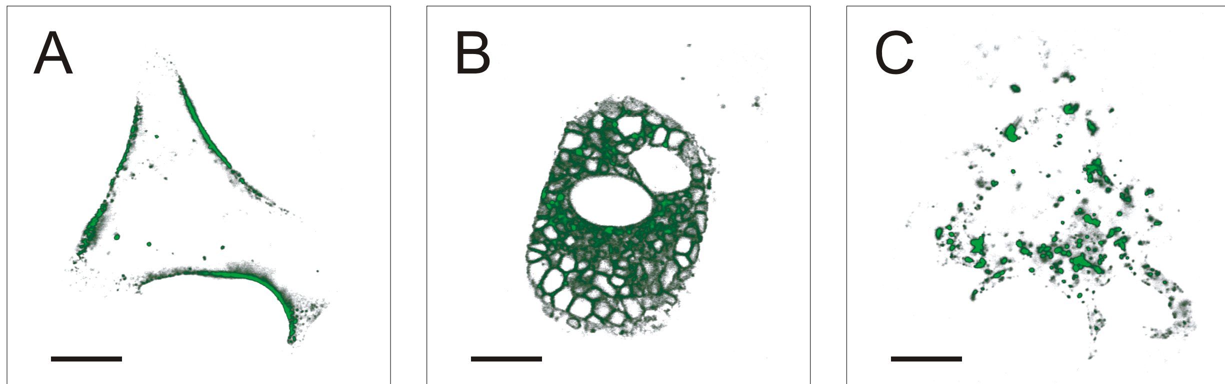

Figure 5.8: Plasma membrane trafficking of HB–EGF in VSM cells. A–C, VSM cells were nucleofected with an expression plasmid encoding a C–terminally YFP–fused HB–EGF construct and seeded on poly–L–lysine–coated glass coverslips . 1 h after the nucleofection, cells were treated with 7 μM brefeldin A (B) or 2 μM cytochalasin D (C) for 4 h as indicated, and then imaged by confocal laser scanning microscopy (Zeiss LSM–510META, equipped with an α–Plan Fluar 100x/1.45 objective). YFP was excited at 488 nm and emitted light was detected through a 505–nm long pass filter. Scale bar, 10 μm.

5.6 The localization of the newly synthesized HB–EGF in the plasma membrane correlates with the second phase of the ERK1/2 phosphorylation

Since the expression of the fluorescent fusion protein HB–EGF–YFP is not regulated by the HB–EGF promotor, the experiments described in the previous section to elucidate the trafficking of HB–EGF only indicated that the fluorescent fusion protein HB–EGF–YFP is rapidly processed and transported to the plasma membrane. To demonstrate that *de novo* insertion of endogenously synthesized HB–EGF into the plasma membrane coincides with the time course of the thrombin–induced long–lasting ERK1/2 phosphorylation, a membrane preparation of VSM cells was performed. VSM cells were seeded in 66–mm culture dishes, maintained in serum–free medium for 36 h and stimulated with thrombin (2 U/ml) for up to 240 min. Membrane preparation was performed as explained in *Material* and *Methods* sections.

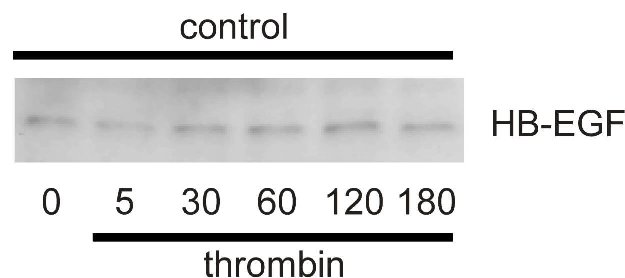

Figure 5.9: Membrane localization of endogenous HB–EGF in VSM cells. Membrane preparations were obtained from thrombin–stimulated VSM cells. Membrane samples were subjected to 12% SDS–PAGE, electroblotted, and probed with anti–HB–EGF.

If *de novo* synthesis and membrane insertion would limit the EGFR transactivation and subsequent ERK1/2 phosphorylation, an initial depletion of HB–EGF should be followed by replenishment with newly synthesized protein. Indeed, an early loss of HB–EGF in membrane preparations was discernible 5 min after VSM cell stimulation (Fig. 5.9). Additionally, a resupply of HB–EGF in the plasma membrane is observed 60 min after

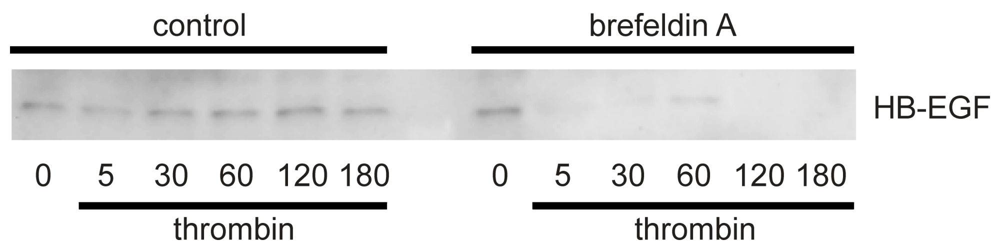

Figure 5.10: Brefeldin A inhibits the replenishment of HB–EGF into the plasma membrane in VSM cells. Membrane preparations were obtained from thrombin–stimulated VSM cells with or without pre–incubation with brefeldin A (7 μM added 30 min before thrombin stimulation), and probed with an anti–HB–EGF antibody. Note the rapid decrease of HB–EGF expression in the presence of brefeldin A.

thrombin stimulation. The results coincide closely with the first shedding of HB–EGF which leads to the first phase of ERK1/2 activation, and the replenishment of the protein into the plasma membrane for the second wave of the thrombin–induced ERK1/2 phosphorylation.

To further support the effect of brefeldin A in the trafficking of HB–EGF,treatment of VSM cells with brefeldin A, subsequent thrombin stimulation and membrane preparation was performed. To this end, the cells were pretreated with 7 μM brefeldin A added 30 min before thrombin stimulation and then prepared as described before. The presence of brefeldin A completly abolished the HB–EGF expression or its transport between the ER and the Golgi apparatus. Thus, brefeldin A prevents the replenishment of HB–EGF into the plasma membrane after the first shedding of HB–EGF. HB–EGF expression did not recover from this initial loss, and remained low, indicating that disruption of ERK1/2 signaling by brefeldin A may be caused by a limiting amount of cleavable HB–EGF in the plasma membrane (Fig. 5.10).

5.7 A triple membrane–passing signaling mechanism and EGFR activation lead to the delayed ERK1/2 activation by TRAP stimulation in VSM cells

The molecular mechanisms of the GPCR–induced EGFR transactivation remained elusive for a long time. Nowadays, multiple results indicate that, upon GPCR stimulation, an MMP–mediated shedding of EGFR ligands leads to activation and autophosphorylation of the EGFR, resulting in the activation of the canonical Ras/Raf/MEK/ERK pathway. Thrombin stimulation activates three different receptors; PAR1, PAR3 and PAR4. To define the details of the PAR1–induced second phase of ERK1/2 activation, VSM cells were stimulated with the thrombin receptor agonist peptide (TRAP), which is a selective PAR1–activating peptide and, similar to thrombin, induces a biphasic ERK1/2 phosphorylation.

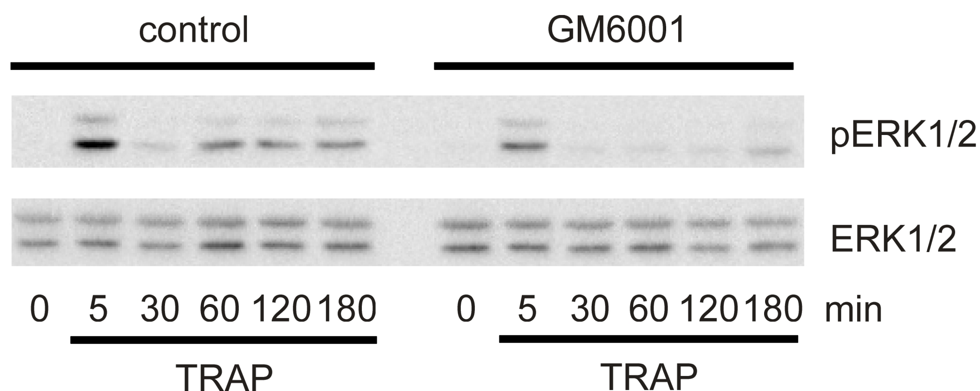

Figure 5.11: ERK1/2 activation in TRAP-stimulated VSM cells requires MMP activity. Serum–starved cells were stimulated with 25 mM TRAP in the absence or the presence of 10 mM GM6001 (added 30 min prior to TRAP stimulation). Aliquots of whole–cell lysates were subjected to 10% SDS–PAGE, electroblotted and probed with anti–phospho–ERK1/2 or anti–ERK1/2 antibodies as indicated. A representative example of three independent experiments is shown.

5.7.1 TRAP–induced long–lasting of ERK1/2 phosphorylation requires the shedding of EGFR ligands by MMPs

To test whether the PAR1–induced second phase of ERK1/2 phosphorylation requires MMP activity, VSM cells were pretreated with GM6001, a potent broad–spectrum inhibitor of MMPs and ADAMs. Serum–starved cells were pretreated (30 min prior to TRAP stimulation) with GM6001 (10 mM) and then stimulated with 25 mM TRAP. Western blot analysis of ERK1/2 phosphorylation in whole–cell lysates revealed that, in presence of GM6001, the late phase of ERK1/2 activation is almost blunted (Fig. 5.11). In addition, the first phase of ERK1/2 activation is attenuated to approximately 50%, indicating a role of the shedding of EGFR ligands not only for the signaling of the second wave of ERK1/2 activation but also for the initial signaling towards ERK1/2. Since the

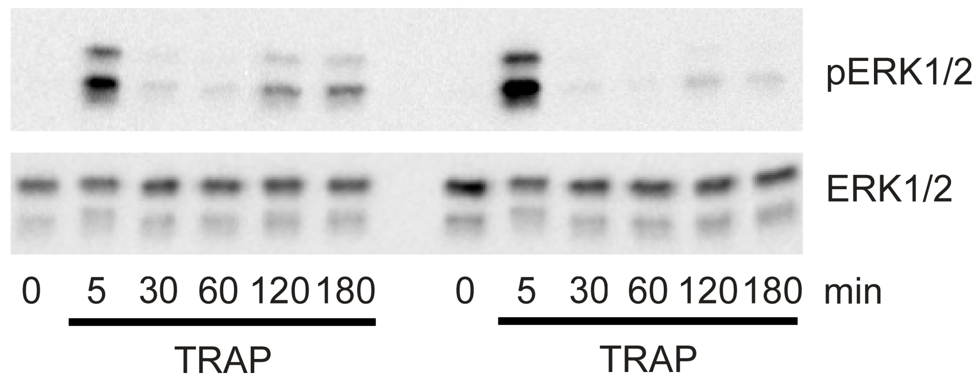

Figure 5.12: The long–lasting ERK1/2 activation by TRAP stimulation in VSM cells requires the shedding of EGFR ligands. Serum–starved cells were stimulated with 25 mM TRAP and treated with 10 mM GM6001 30 min after TRAP stimulation. Whole–cell lysates were subjected to 10% SDS–PAGE, electroblotted and probed with anti–phospho–ERK1/2 or anti–ERK1/2 antibodies as indicated.

first phase of the ERK1/2 activation is reduced by GM6001 treatment,a possible interpretation of the inhibition of the second phase of ERK1/2 phosphorylation is that the EGFR transactivation during the early phase of the ERK1/2 activation is required to stimulate *de novo* expression of some protein, probably HB–EGF, that mediates the late phase of the ERK1/2 phosphorylation. To test if the TRAP–induced long–lasting ERK1/2 activation

depends on the shedding of newly synthesized HB–EGF, cells were treated with GM6001 only 30 min after TRAP stimulation.

Under these conditions, the early phase was allowed to occur without inhibition. In this case, the second phase of the ERK1/2 activation by TRAP stimulation was still suppressed (Fig. 5.12), indicating that indeed a sustained shedding activity is required for triggering the delayed and long–lasting PAR1–mediated ERK1/2 phosphorylation in VSM cells.

5.7.2 The second phase of the ERK1/2 activation in response to TRAP necessitates the activation of the EGFR

To test whether the signal transmission which leads to the long–lasting ERK1/2 phosphorylation by TRAP stimulation needs a ligand–induced EGFR autophosphorylation, VSM cells were treated with AG1478. The tyrphostin AG1478 is an EGFR blocker, which

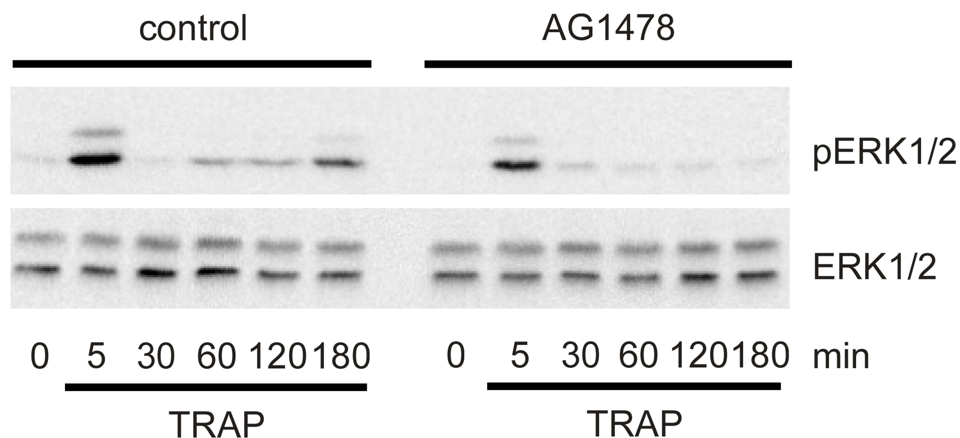

Figure 5.13: ERK1/2 phosphorylation requires the activation of the EGFR. VSM cells were incubated for 36 h in serum–free medium, pretreated with 250 nM AG1478 for 30 min, and subsequently stimulated with 25 mM TRAP. Cell lysates were separated on a 10% SDS–PAGE, electroblotted and probed with anti–phospho ERK1/2 and anti–ERK1/2.

inhibits the autophosphorylation of the receptor after ligand binding. VSM cells were maintained in serum–free medium for 36 h and pretreated with 250 nM AG1478 30 min prior to TRAP stimulation (25 mM). Analysis of whole cell lysates from AG1478–treated cells displayed a similar pattern of ERK1/2 phosphorylation as GM6001–treated cells after

TRAP stimulation. The inhibition of the EGFR autophosphorylation completly abolished the second phase of the ERK1/2 activation (Fig. 5.13) whereas the first phase was only reduced to approximately 50%. This indicates that EGFR activation occurs in both phases of the ERK1/2 phosphorylation by TRAP stimulation.

To demonstrate that the inhibition of the second phase of the ERK1/2 phosphorylation is not an effect of the reduction of the first phase of the ERK1/2 activation, serum–starved VSM cells were treated with AG1478 (250 nM) 30 min subsequent to TRAP stimulation, allowing the early phase of ERK1/2 activation to occur. Figure 5.14 showns that the late phase of ERK1/2 phosphorylation is also completely abrogated by delayed blocking of the EGFR activation. These results confirmed that the PAR1–mediated late phase of ERK1/2 activation is a process which requires the shedding of EGFR ligands by MMP and the activation of the EGFR for triggering the differentiation signal.

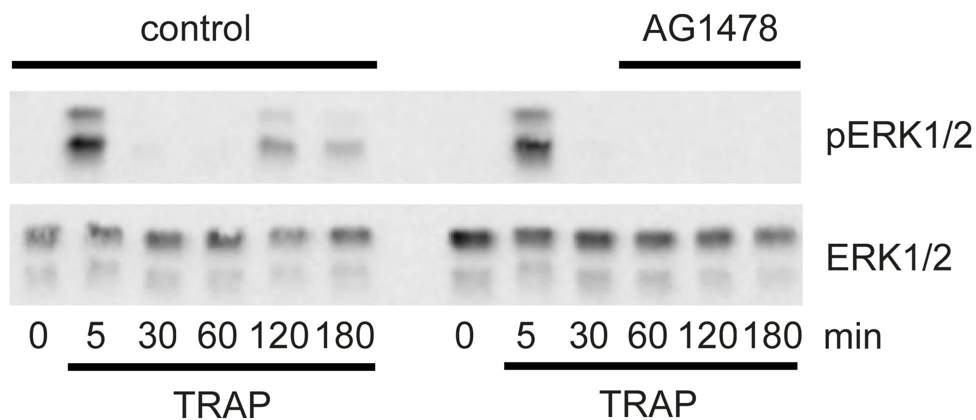

Figure 5.14: The late phase of ERK1/2 requieres the activation of the EGFR. Serum–starved VSM cells were stimulated with 25 mM TRAP and treated with 250 nM AG1479 30 min later. Whole–cell lysated were subjected to 10% SDS–PAGE, electroblotted and probed with anti–phospho–ERK1/2 or anti–ERK1/2 antibodies.