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PAP1 signaling involves MAPK signal transduction

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Abstract Pancreatitis-associated protein 1 (PAP1) belongs to the Reg family of secretory proteins. Several important biological roles have been attributed to PAP1 but the signaling pathways activated by this protein remain only partially understood. Here, we describe the intracellular pathways triggered by PAP1 in a pancreatic acinar cell line. Taking advantage of the fact that PAP1 induces its own transcription, we performed ChIP assays to analyze the recruitment of transcriptional factors on its promoter. Our results show that PAP1 increased the transactivation activity of *pap1* and the binding on its promoter of the nuclear factors C/EBPβ, P-CREB, P-ELK1, EGR1, STAT3, and ETS2, which are downstream targets of MAPK signaling. p44/42, p38, and JNK MAPKs activity increased after PAP1 treatment. In addition, pharmacological inhibition of these kinases markedly inhibited the induction of *pap1* mRNA. Taken together, these results indicated that the mechanism of PAP1 action involves the activation of the MAPK superfamily.

Introduction

Pancreatitis-associated protein 1 (PAP1) is a C-type lectin originally identified as a pancreatic secretory protein, not expressed under physiological conditions but strongly overexpressed during acute pancreatitis [1]. Its expression is actually not restricted to the pancreas and has been linked to a variety of diseases including inflammatory bowel disease [2], Alzheimer's disease [3], or some types of cancers such as hepatocellular carcinoma [4], pancreatic adenocarcinoma [5], and colorectal carcinoma [6].

PAP1 is a 175-amino-acid-long polypeptide containing one carbohydrate-binding domain (CBD) linked to an N-terminal sequence, part of which is cleaved during its maturation and secretion [7]. This protein was the first identified member of a family of proteins that comprises lithostatine and several isoforms of PAP sharing important sequence similarities [8]. Data from experimental acute pancreatitis showed that the secretory patterns of these proteins are coordinated, with a peak of expression in pancreatic tissue or juice 24 h after the induction of acute pancreatitis by cerulein [9]. However, the levels and the expression pattern of these proteins are different, suggesting that these isoforms do not substitute one another but may have different functional properties [10].

PAP1 has been described in different species under different names: it was called p23 protein when secreted by rat pituitary cells [11], HIP/PAP when reported in hepatocellular cancer [4], or Reg2 when expressed in regenerating motor neurons [12]. Also, PAP1 was shown to be involved in several biological functions, sometimes

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apparently unrelated. In pancreatic acinar cells, overexpression of PAP1 increases resistance to apoptosis [13, 14]. PAP1 acts as a growth factor promoting cell proliferation in hepatocytes [15] and motor neurons [12]. Moreover, PAP1 exhibits mitogen activity on Schwann cells and promotes nerve regeneration [16]. It also promotes intestinal epithelial cell growth [17]. A function linking cell migration and cell adhesion to extracellular matrix has also been proposed for PAP1 [18, 19].

Widening the PAP1 range of functions, our group demonstrated an anti-inflammatory role for this protein in agreement with its strong induction during the course of inflammatory diseases such as pancreatitis, Crohn's disease, and ulcerative colitis. In a rat model of acute pancreatitis, the infusion of anti-PAP1 antibodies worsened the pancreatic inflammatory response [20]. In the same study, PAP1 treatment prevented TNFα-induced Nuclear Factor kappa B (NFκB) activation in macrophages. This protective function of PAP in acute pancreatitis has been confirmed using antisense PAP oligonucleotides in order to block the expression of all three isoforms of PAP [21]. In addition, Gironella et al. [22] corroborated the anti-inflammatory role of PAP1 in vivo in a PAP1-deficient mice model.

Although a number of studies have focused on the physiopathological significance of PAP1, little is known of the signal transductions triggered by PAP1. Recent data have shown that PAP1 inhibited the inflammatory response through a JAK/STAT3-dependent mechanism [23]. Nevertheless, PAP1 effects are not entirely explained by the activation of JAK/STAT3 as it is known that signals transduction usually involves an intricate and complex network of interactions between several signaling pathways. The purpose of this study was to identify the intracellular signaling pathways induced by PAP1. Taking advantage of the fact that PAP1 induces the expression of its own gene, we performed a Chromatin Immunoprecipitation assay (ChIP) to correlate transcriptional factor recruitment to the *pap1* promoter with the signaling pathways triggered by PAP1. Our results showed that PAP1 increased the transactivation activity of the nuclear transcriptional factors associated with the MAPK superfamily.

Materials and methods

Reagents

All culture supplies were obtained from Invitrogen (Carlsbad, CA). Reagents for SDS-PAGE, blotting paper and nitrocellulose membranes were from BioRad (Hercules, CA). Antibodies against P-p38, P-p44/42 and P-JNK were supplied by Cell Signaling Technology (Beverly, MA).

Antibodies against β-actin, C/EBPβ, P-CREB, P-ELK1, ATF2, EGR1, c-JUN, STAT3, NFκB (p65), ETS2, ATF3, SRF, and RNApol II were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies linked to horseradish peroxidase were purchased from Sigma (St. Louis, MO). Specific inhibitors for each MAPK families: SB203580 (p38 MAPK inhibitor), PD98059 (p44/42 inhibitor), and JNK Inhibitor II (JNK inhibitor) were obtained from Calbiochem (Nottingham, UK). Recombinant human PAP1 was produced in the yeast *Pichia pastoris* according to the recommendations of the manufacturer by Dynabio (Marseille, France). The PAP1 ELISA used to quantify PAP1 (PancrePAP) was obtained from Dynabio.

Cell culture

Rat pancreatic acinar cells (AR42J) were grown in monolayers until 70–80% confluence in a Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum 'Gold' (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 100U/mL penicillin and 100 μg/ml streptomycin (Sigma) at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cultures were split every 3 days by trypsinization with 0.1% trypsin (Sigma) in Ca^{2+}/Mg^{2+}-free PBS containing 0.9 mM EDTA.

Chromatin immunoprecipitation assay and RNApol ChIP

ChIP and RNApol ChIP procedures were performed according to Sandoval and colleagues [24], and Rodriguez and colleagues [25] with some modifications. AR42J cells were fixed by adding formaldehyde to a final concentration of 1% directly to the cell culture dish, and incubating for 7 min on a shaking platform, at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M, and cells were then washed twice with ice-cold PBS, scrapped and recovered by centrifugation at 3,000g for 5 min. The pelleted cells were resuspended in 6 ml of cell lysis buffer (HEPES 5 mM, pH 8, KCl 85 mM, NP40 0.5%) supplemented with protease inhibitor cocktail (Sigma), incubated on ice for 15 min and centrifuged at 1,000g for 5 min. The nuclei pellet was resuspended in 1 ml of nuclei lysis buffer (Tris–HCl 50 mM, pH 8.1, EDTA 10 mM, SDS 1%) and stored at −80°C until use.

Cross-linked chromatin was sonicated on ice during 8 pulses of 10 s each, with a 37% amplitude on a Vibra-Cell VCX-500 sonicator (Sonics and Materials, Newtown, CT) to obtain chromatin fragments of 300–500 bp. This target size of chromatin fragments was confirmed by agarose gel electrophoresis. The sonicated chromatin was centrifuged at 14,000g for 10 min, and the supernatants were diluted

10-fold with dilution buffer (NaCl 165 Mm, SDS 0.01%, Triton X-100 1.1%, EDTA 1.2 mM, Tris–HCl 16.7 mM, pH 8.0) supplemented with protease inhibitor cocktail (Sigma). Aliquots from the diluted supernatants (equivalent to 50 μg DNA) were taken and incubated for 2 h at 4°C with Dynabeads-protein G (Invitrogen) previously washed with PBS/BSA (5 mg/ml) and saturated with tRNA from E. coli and 2 μg of the corresponding antibody on a rotating platform (C/EBPβ, P-CREB, P-ELK1, ATF2, EGR1, c-JUN, STAT3, NFκB (p65), ETS2, ATF3, and SRF for ChIP assay, or RNApol II for RNApol ChIP). The chromatin fragment/antibody/protein G-Dynabead immunocomplexes, were recovered applying a magnetic field, by placing the sample containing tubes in the appropriate magnet (Invitrogen). Immunocomplexes were washed twice with ice-cold low-salt buffer (HEPES 50 mM, pH 8.0, NaCl 140 mM, Triton X-100 1%, sodium deoxycholate 0.1%, EDTA 1 mM), twice with ice-cold high-salt buffer (HEPES 50 mM, pH 8.0, NaCl 500 mM, Triton X-100 1%, sodium deoxycholate 0.1%, EDTA 1 mM), twice with ice-cold LiCl buffer (Tris–HCl 10 mM, LiCl 250 mM, NP-40 0.5%, sodium deoxycholate 0.5%, EDTA 1 mM) and finally once with TE buffer. During each washing step, the Dynabead suspensions were kept under rotation for 4 min at 4°C, and recovered with the magnet. After the elimination of any remaining TE buffer from the last wash, the immunoselected chromatin was eluted from the Dynabead-protein G in two consecutive steps, by adding 50 μl of elution buffer (Tris–HCl 50 mM, EDTA 10 mM, SDS 1%), vortexing and incubating for 10 min at 65°C. The resulting 100 μl fraction (IP fraction) was incubated at 65°C overnight to reverse formaldehyde crosslinks, in the presence of proteinase K (0.4 mg/ml). An

aliquot of the cross-linked and diluted chromatin was treated in parallel as above, but in the absence of antibody (NoAb fraction), and the first supernatant, resulting from putting the sample on the magnet prior to the washing steps, was saved as the Input fraction. The DNA from all these samples (IP, NoAb and Input) was purified with a PCR purification kit (Qiagen, Valencia, CA) and used for PCR analysis of the target gen.

PCR analysis of immunoprecipitated chromatin

Input, IP, and (NoAb) fractions were analyzed by PCR with the appropriate primer pairs to amplify products of 200–300 bp in length, corresponding to either the promoter or the coding regions of the target gene. For the analysis, 1:500 dilutions of the Input and 1:30 of the IP and NoAb fractions were used. Primers were designed to amplify rat *pap1* promoter and rat *pap1* coding region. PCR fragments were size-fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed with FLA3000 electronic autoradiography system (Fujifilm, Japan). Analysis of constitutive expressed β-actin gene was used as a positive control and analysis of an intergenic region was used as a negative control. Sequence primers are showed in Table 1.

RNA isolation and RT-PCR

Analysis of the expression of *pap1* was conducted using a semiquantitative RT-PCR method. Total RNA from cells was extracted using the Trizol reagent (Invitrogen). One μg total RNA was used for amplification using the Promega AccessQuick RT-PCR System (Promega, Madison, WI)

Table 1 Primers used for RT-PCR and real time PCR

Name	Primer
Rat *pap1* (promoter)	Forward: 5′-ATGCCACCATTTTTTCCTTGTG-3′
	Reverse: 5′-GAT GGGAAGCACTTGGCATT-3′
Rat *pap1* (coding region)	Forward: 5′-CAAAGGCTCCC AGGCATATG-3′
	Reverse: 5′-GGCCAGCTATGTGAGGAACAA-3′
Rat *pap1* (real time)	Forward: 5′-ACTCCATGACCCCACTCTTG -3′
	Reverse: 5′-TGACAAGCTGCCACAGAATC -3′
Rat β-actin (coding region)	Forward: 5′-AGAGCAAGAGAGGCATCCTG-3′
	Reverse: 5′-GGGTCATCTTTTCACGGTTGG-3′
Rat *gapdh* (real time)	Forward: 5′-GTGTGTCTTTCCGCTGTTTTC -3′
	Reverse: 5′-TGTGCTGTGCTTATGGTCTCA 3′
Rat *pap1* (RT-PCR)	Forward: 5′-TGACAAGCTGCCACAGAATC-3′
	Reverse: 5′-GCTCCTACTGCTATGCCCTG-3′
Rat *gapdh* (RT-PCR)	Forward: 5′-ATGGGAAGCTGGTCATCAAC-3′
	Reverse: 5′-CCACAGTCTTCTGAGTGGCA-3′
Rat intergenic	Forward: 5′-TGCCGGTTATCACTCTCTCATGC-3′
	Reverse: 5′-GGTCTTGGTTCAGTCTTCACATGC-3′

according to the manufacturer's instructions. The following primers were used: rat *pap1* and rat *gapdh* as internal control for stable expression (housekeeping gene). Respective sequence primers are in Table 1. Fragments were amplified using 25–30 cycles of PCR, each cycle consisting of 15 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The resulting RT-PCR products were electrophoresed on 2% agarose gels with DNA markers, stained with ethidium bromide, and visualized under UV light.

Real-time quantitative PCR

Total RNA was isolated from AR42J cells exposed to PAP1 for 20 min and from non-treated control cells, using the Trizol reagent (Invitrogen). The RNA was quantified by measurement of the absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Reverse transcriptase real-time quantitative PCR was performed using an ABI Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA). RT-PCR mixtures contained 0.5 μg of total RNA and 200 nM each of the forward and reverse primers. Primers were designed to amplify rat *pap1* and rat *gapdh*. Respective sequence primers are showed in Table 1. Reaction mixtures also contained Hot-Start Taq DNA polymerase, DNA double-stranded specific SYBR Green I dye, OmniScript and SensiScript reverse transcriptases, ROX passive dye, and nucleotides (Quantitect SYBR Green RT-PCR kit; Qiagen). Amplification was achieved following an initial incubation at 50°C for 30 min, followed by denaturation at 95°C for 15 min, and then 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. At the end of the 40 cycles, samples were subjected to melting analyses to confirm the specificity of PCR products. The fold induction of *pap1* mRNA was determined by the ΔCt relative quantification method (Applied Biosystems). The amount of mRNA in each sample was normalized to the level of *gapdh* in cognate samples.

Chloramphenicol Acetyltransferase gene reporter assay and cell transfection

The rat *pap1* gene promoter fragment including nucleotides −1253 to +10 was inserted upstream of the Chloramphenicol Acetyltransferase (CAT) gene in the pCAT-Basic vector (Promega) as previously reported [26]. AR42J cells (3×10^5 per plate) were plated in 50-mm-diameter culture plates 24 h before transfection. For each plate, 1 μg of the reporter vector (−1,253 to +10-PAP1-CAT) and 1 μg of pCMV/β-gal were added to 6 μl of FuGENE 6 according to the instructions of the supplier (Roche, Palo Alto, CA). The mixture was added to Dulbecco's modified Eagle's medium containing 3 ml of 10% (w/v) FCS. After 24 h in culture, cells were treated with 500 ng of PAP1 alone or in combination with SB203580 (10 μM), PD98059 (100 μM) or the JNK Inhibitor II (100 μM) for an additional 24 h-period. Cell extracts were prepared using the reporter lysis buffer (Promega). CAT activity was determined using the phase-extraction procedure [27], and the β-galactosidase assay was performed essentially as described previously [26]. CAT activity was normalized to the β-galactosidase activity. CAT and β-galactosidase activities were always within linear range of the assay.

SDS-PAGE and Western blot

Cells were lysed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) under conditions for preparation of cytoplasmatic and nuclear extracts. SDS-PAGE was performed on 10% acrylamide gels. Proteins were electrotransferred to nitrocellulose membranes and probed with primary antibody (anti-P-p38 1/1000, anti-P-p44/42 1/1000, anti-P-JNK 1/1000, anti-β-actin 1/1000). The membranes were incubated with the corresponding peroxidase-conjugated secondary Ab, washed, and then incubated with ECL reagents from Bio-Rad before exposure to high performance chemiluminescence films. Gels were calibrated using Bio-Rad standard proteins with markers covering a 7–240 kDa range.

Bioinformatic analysis of *pap1* promoter region

pap1 promoter sequence (1,000 bp) was analyzed using the transcription factor binding sites (TFBS) analysis tool MatInspector (http://www.genomatix.de/) [28].

Statistical analysis

Data were expressed as mean ± SEM. Mean of values from different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed for evaluation the significance of differences between groups. Differences were assumed to be significant when $P < 0.05$.

Densitometric analysis

ImageJ 1.32 software (obtained from the http://rsbweb.nih.gov/ij/download.html) was used to quantify the intensities of the bands obtained in PCR experiments.

Results

Induction of *pap1* gene expression in response to PAP1

We confirmed our previous results showing the positive feedback induction of PAP1 in AR42J pancreatic acinar
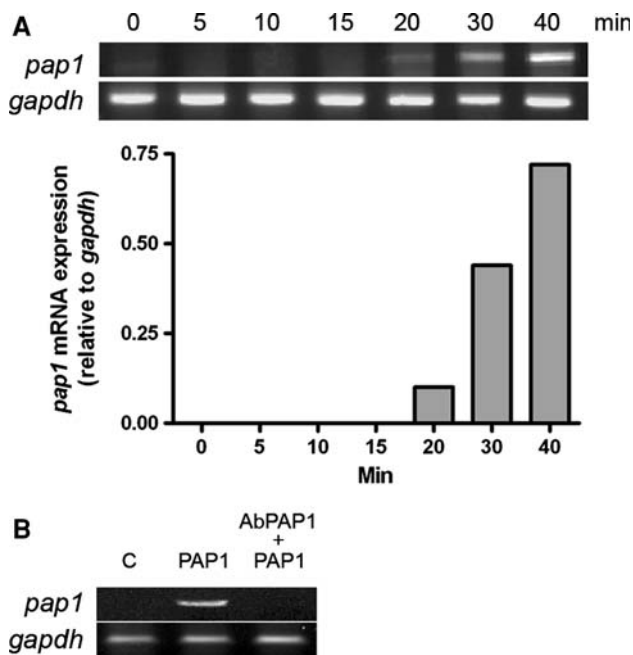
Fig. 1 Time course of *pap1* mRNA expression in response to PAP1. AR42J cells were stimulated with 500 ng PAP1/mL culture medium at different time-points (**a**) or with a mixture of PAP1 plus a blocking anti-PAP1 antibody for 40 min (**b**). Total RNA was extracted and subjected to RT-PCR using specific primers for *pap1* and *gapdh* (internal control). Products were analyzed by 2% agarose ethidium bromide gel. Results are shown for one of three representative experiments

Fig. 2 RNApol ChIP analysis of the rat *pap1* transcription at different times of PAP1 treatment. The cross-linked chromatin samples were immunoprecipitated with an anti-RNA polymerase II antibody and the immunoprecipitates were analyzed by PCR using primers specific for the *pap1* coding region. For each time-point, a sample of total chromatin (*Input*) was included in the PCR. The presence of RNA polymerase in these regions is taken as a measure of actual transcription. Constitutive expressed *β-actin* gene was used as a positive control and an intergenic region was used as a negative control. *No Ab* No antibody

cells. AR42J cells displayed a time-dependent induction of pap1 gene expression after adding PAP1 to the culture media. Kinetic analysis of the induction revealed that the levels of *pap1* mRNA increased 30 min after addition of PAP1 at 500 ng/ml (Fig. 1a). This dose has been previously reported to be effective in inducing PAP1 itself [23]. Specificity of PAP1-induced response was verified by incubating the protein with 1 µM of rabbit anti-PAP1 neutralizing antibody [20] for 30 min. Then, AR42J cells were stimulated with this mixture for 40 min. Figure 1b shows the effective neutralizing ability of anti-PAP1 antibody on PAP1 induction. These results allowed us to use the *pap1* gene promoter as a strategy to study the signaling pathways activated by PAP1 using ChIP and RNApol ChIP assays.

Identification of transcription factors bound to the *pap1* gene promoter in vivo

To verify the binding of regulatory factors to the *pap1* promoter in response to PAP1, we carried out a ChIP assay in growing AR42J cells. After cross-linking and immuno-precipitation, enrichment of the human *pap1* promoter was monitored by PCR amplification using specific primers for the promoter region. In order to verify the results obtained, the same samples employed for the ChIP assay were used
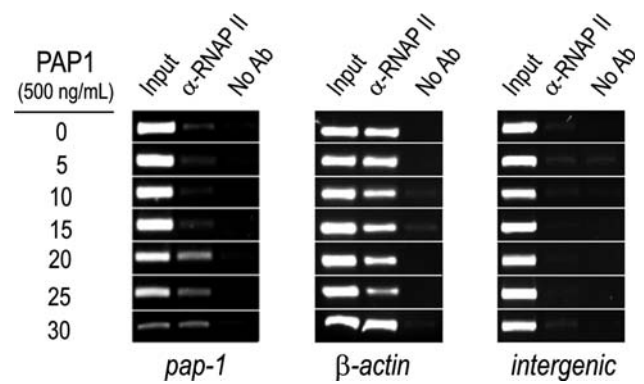
for the RNA polymerase ChIP assay, as previously described. The α-RNA polymerase antibody precipitated both promoter and coding chromatin fragments, thus confirming that the *pap1* gene is expressed in AR42J cells. An augmented transcription was observed 20 min after PAP1 addition to the cell culture medium (Fig. 2). On that basis, we established an incubation time of 20 min to test the binding of nuclear regulatory factors to the *pap1* promoter region. In vivo binding of factors to *pap1* promoter was monitored using antibodies against C/EBPβ, P-CREB, P-ELK1, ATF2, EGR1, c-JUN, STAT3, NFκB (p65), ETS2, ATF3, and SRF. Figure 3 depicts the presence of downstream targets of PAP1. EGR1, and STAT3 bound to the promoter region of *pap1* gene in cells without PAP1 treatment. After stimulation with PAP1, the binding of C/EBPβ, P-CREB, P-ELK1, EGR1, STAT3, and ETS2 was dramatically increased, while that of ATF2, c-JUN, NFκB (p65), ATF3, and SRF remained unchanged.

PAP1 signal transduction involved MAPK signaling pathway

Next, we investigated whether PAP1 directly affect MAPK signaling. We used Western blot and specific antibodies against the active (phosphorylated) forms of the kinases. As summarized in Fig. 4, AR42J cells presented basal levels of phosphorylation of the three members of the MAPK superfamily: p44/42, p38, and JNK. However, 30 min after PAP1 treatment, an augmented activation of the p44/42, p38 and JNK protein kinases was observed, suggesting that PAP1 signal transduction involves the MAPK superfamily.
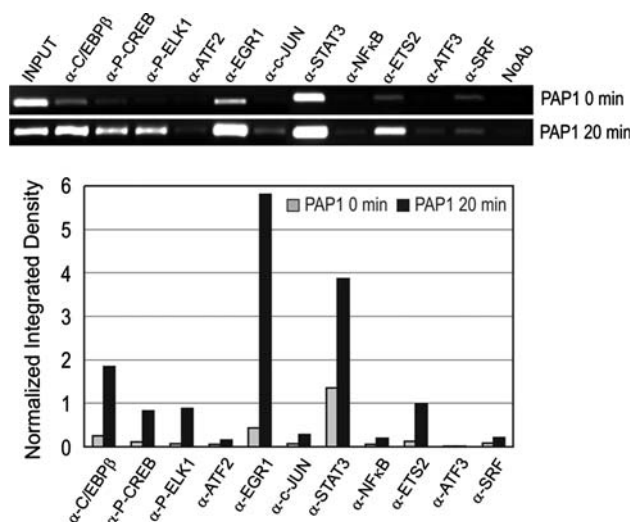
Fig. 3 ChIP analysis of *pap1* promoter occupation during PAP1-treated AR42J cells. ChIP analysis was performed to monitor the binding of nuclear factors within the PAP1 promoter. In vivo binding of factors to *pap1* gene 20 min after PAP1 treatment was monitored using antibodies against C/EBPβ, P-CREB, P-ELK1, ATF2, EGR1, c-JUN, STAT3, NFκB (p65), ETS2, ATF3, and SRF. The immuno-precipitated samples were analyzed by PCR using primers of the *pap1* promoter region. The normalized integrated density of the immuno-precipitated samples bands was included as a quantification of the relative intensity of the transcriptional binding. Results are shown for one of three representative experiments

Effect of MAPKs specific inhibitors on PAP1-mediated induction of *pap1* mRNA

Given the fact that PAP1 enhanced the phosphorylation of MAPK family members, we examined the effect of MAPK specific inhibitors on PAP1-mediated induction of *pap1* mRNA. Pancreatic acinar cells were treated for 20 min with vehicle, PAP1 (500 ng/ml) or pre-treated with different MAPK specific inhibitors: SB203580 (p38 MAPK inhibitor; 100 μM), PD98059 (p44/42 inhibitor; 100 μM), and JNK Inhibitor II (100 μM) for 30 min, and then stimulated with PAP1. As shown in Fig. 5, the pharmacological inhibition of the three main MAPK family members markedly inhibited the activation of *pap1* mRNA, thus indicating that activation of the MAPK superfamily is essential for *pap1* mRNA synthesis in response to PAP1. In order to verify the effect of MAPK inhibition on the activation of the *pap1* promoter, an additional experiment was carried out using a gene reporter assay. Figure 6 shows that all inhibitors resulted in a blockage of the CAT activity induced by PAP1.

Discussion

A plethora of functional roles have been attributed to the stress-induced secretory protein PAP1. This protein has
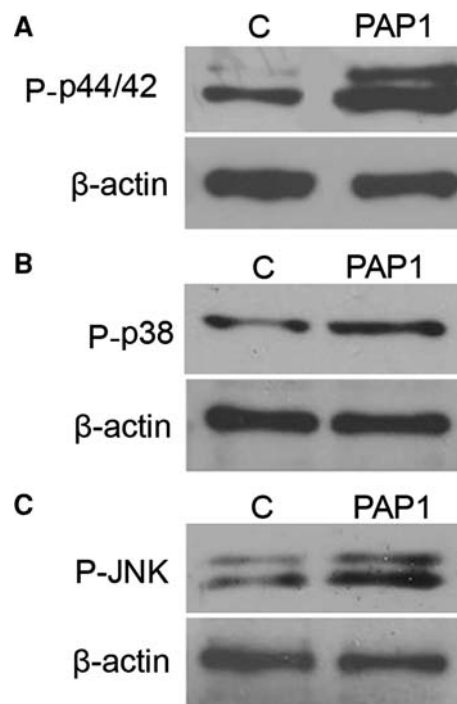

Fig. 4 Effect of PAP1 on MAPKs activity. Phosphorylation levels in response to PAP1 of the three main members of the MAPK superfamily were assessed by Western blot. AR42J cells were stimulated with or without 500 ng/ml PAP1 for 30 min and cell lysates were subjected to immunoblotting using anti-P-p44/42 (**a**), anti-P-p38 (**b**) and anti-P-JNK (**c**) antibodies. The presence of PAP1 enhanced the expression of phosphorylated, active forms of the three MAPKs. The same membranes were re-blotted for anti-β-actin to check loads were similar. Immunoblots shown are representative for one of three independent experiments

been reported to be mitogenic, anti-apoptotic, or anti-inflammatory, to regulate cellular adhesion, promote bacterial aggregation, or increase resistance to anti-tumoral agents, the nature of these functions depending on the cell type used as model and the environment.

Since different investigators have attributed diverse physiological roles to PAP1, little is known about the downstream signaling pathways activated by PAP1. To date, it was shown that, in cultured motor neurons, PAP1 signals for survival through the phosphoinositol-3-kinase (PI3 kinase)/AKT kinase pathway [12]. In hepatocytes, PAP1 stimulates PKA-dependent phosphorylation of the pro-apoptotic Bad protein, suggesting that PAP1 may compete with cAMP to stimulate PKA activity [15]. Moreover, recent data have shown an anti-inflammatory role for PAP1 through the inhibition of NFκB signaling induced by TNFα in pancreatic acinar cells [23]. This inhibition takes place as a result of JAK/STAT3 activation suggesting the existence of a crosstalk between both signaling pathways.

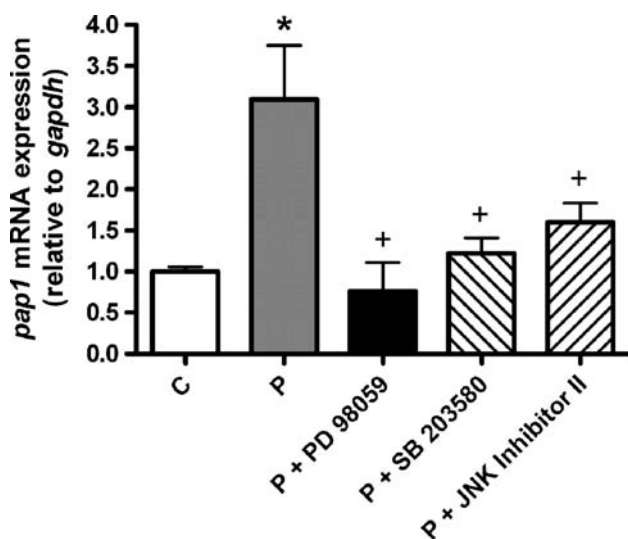Despite this well-documented data, they can barely account for all PAP1 functions. In an effort to complete the

Fig. 5 Effect of MAPK specific inhibitors on PAP1-mediated induction of *pap1* mRNA. AR42J cells were treated for 20 min with vehicle, PAP1 (500 ng/ml) or pre-treated with different MAPK specific inhibitors: p44/42 inhibitor (SB203580; 100 μM), p38 inhibitor (PD98059; 100 μM), and JNK inhibitor (JNK Inhibitor II; 100 μM) for 30 min before stimulation with PAP1. *pap1* mRNA induction levels were quantified by real-time PCR and normalized to *gapdh* mRNA expression. p38, p44/42, and JNK MAPKs inhibitors decreased *pap1* mRNA induction. Results are expressed as mean values ± SEM. *$P < 0.05$ vs C, +$P<0.05$ vs P. *C* Control, *P* PAP1. Results are shown for one of three representative experiments

Fig. 6 Transcription activation of the *pap1* promoter by PAP1 is inhibited by MAPK inhibitors SB203580, PD98059, and the JNK Inhibitor II. AR42J cells were transiently transfected with the −1,253 to +10-PAP1-CAT construct together with pCMV/β-gal vector used as an internal control for the transfections. After transfection, the cells were incubated for 24 h and then incubated with 500 ng/ml of PAP1 alone or in combination with p44/42 inhibitor (SB203580; 100 μM), p38 inhibitor (PD98059; 100 μM), and JNK inhibitor (JNK Inhibitor II; 100 μM) by an additional 24 h-period. Cells were then harvested, lysed and assayed for both CAT and β-galactosidase activities. Results are expressed as mean values ± SEM. *$P < 0.05$ vs C, +$P<0.05$ vs P. *C* Control, *P* PAP1. Results are shown for one of three independent experiments

picture, we used a pancreatic acinar cell line AR42J known to respond to PAP1. In previous work on this cell line, we used immunopurified PAP1 from rat pancreatic juice to describe a positive feedback mechanism of PAP1. Here, we show the same results using human PAP1 produced in *Pichia pastoris*, which is not a surprise given the important sequence similarities between the two homologs. Analysis of the kinetics of PAP1 induction revealed that *pap1* mRNA expression analyzed by RT-PCR in response to PAP1 itself was time-dependent (Fig. 1a) and already increased 30 min after the treatment. The specificity of PAP1-induced response was verified using a neutralizing anti-PAP1 antibody (Fig. 1b). These results allowed us to use the ChIP assay on the *pap1* gene promoter to study the signaling pathways activated by PAP1. We therefore tested by RNApol ChIP assay the recruitment of the RNA α-polymerase to *pap1* promoter. As shown in Fig. 2, an enhanced binding of the RNApol II transcriptional complex is observed 20 min after PAP1 addition to the cell culture medium.

Several potential transcription factor binding sites are present on the *pap1* promoter. Analysis of the 1 kb promoter sequence revealed potential binding sites of some important transcription factors, including C/EBP, P-CREB, ATF2, STAT3, ETS2, and SRF. In addition to these, we also evaluated the binding of the nuclear factors P-ELK1,
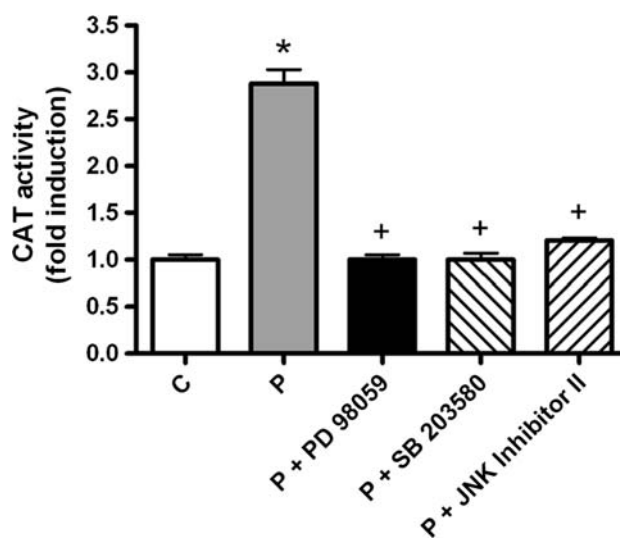
EGR1, c-JUN, NFκB, and ATF3 to study their effects on *pap1* promoter occupation. As shown in Fig. 3, EGR1 and STAT3 are bound to *pap1* promoter in the absence of PAP1 treatment indicating that *pap1* may be considered as a "potentially active" gene prepared to respond promptly to an appropriated cellular stimulus. After stimulation with PAP1, we could obtain significant amplification of the *pap1* promoter region from DNA co-immunoprecipated using the C/EBPβ, P-CREB, P-ELK1, EGR1, STAT3, and ETS2 antibodies, indicating that, in these conditions, these transcription factors are bound to the binding site of *pap1* promoter gene in the cells. In a previous study, we have already reported the PAP1 positive feedback through a JAK/STAT3 mechanism [23]. Therefore, the binding of STAT3 to the PAP1 promoter would be consistent with the STAT3-dependent role of PAP1.

Regarding NFκB, we did not find this nuclear factor bound to the *pap1* promoter. This result is in keeping with previous studies showing no activation of NFκB in pancreatic acinar cells or macrophages in response to PAP1 [2, 23]. However, different results have been found with other PAP isoforms. Using a rat macrophage cell line, Viterbo et al. [29] reported that PAP2, the most expressed PAP isoform during acute pancreatitis, upregulated the expression of pro-inflammatory cytokines IL1, IL6, and

TNFα. These differences seem to be related with the different functional properties shown by the different members of the PAP superfamily [10].

The activation of nuclear factors C/EBPβ, P-CREB, P-ELK1, EGR1, and ETS2 observed in response to PAP1 served as a specific readout for MAPK superfamily activation. Nevertheless, other signaling cascades such as the PI3 kinase/AKT and Ca^{2+}-specific signals activated by growth factors or cellular stress, are known to converge on some of these factors, controlling their activity.

Then, we further delineated the possible influence of PAP1 on the activation of MAPK signaling pathway. A wide variety of extracellular stimuli induce sequential phosphorylation and activation of the MAPK pathway. This superfamily of proteins consists of three main protein kinase families: the extracellular signal-regulated protein kinases (ERK or p44/42), the c-Jun N-terminal kinases (JNK), and the p38 family of kinases. Each molecule is activated by distinct pathways and transmits signals either independently or in coordination [30, 31]. The MAPK pathway is involved in diverse cellular functions including cell proliferation, cell differentiation, and apoptosis as well as inflammation. Thus, it is conceivable that PAP1 triggers the MAPK signaling pathway. In this line, Lieu et al. [32] reported changes in p44/42 activation in PAP1 knockout mice during liver regeneration, suggesting that this MAPK could be involved in the regenerative effects of PAP1.

Western blot analysis of MAPK indicated that AR42J cells presented with a basal level of phosphorylation of p44/42, p38, and JNK (Fig. 4). However, PAP1 treatment enhanced the phosphorylation of all three MAPKs, suggesting that some of the effects of PAP1 could be mediated by activation of MAPK signaling and thus downstream

transcription events. This possibility was evaluated by using specific inhibitors of these three major families of MAPKs and measuring the induction of *pap1* mRNA as well as the activation of *pap1* gene promoter in a gene reporter assay. Results indicated that the addition of p44/42, p38, and JNK pharmacological inhibitors completely blocked the activation of *pap1* promoter as well as the induction of *pap1* mRNA in response to PAP1 (Figs. 5 and 6). It was therefore concluded that these signaling events are necessary to *pap1* gene regulation.

Most experimental evidence obtained so far demonstrates that MAPK signaling cascades are involved in the pathogenesis of acute pancreatitis [33]. An early activation of all three MAPK in the pancreas within minutes was indeed observed in experiments involving various models of experimental acute pancreatitis [34–36]. This is of interest given the fact that our results indicate that PAP1 induces itself in a positive feedback mechanism mediated by MAPKs activation. In these conditions, it is not surprising to find high amounts of this protein during this acute inflammatory disease.

Finally, some reports indicate that MAPK cascades can also influence other signaling pathways. It is known that p44/42 participate in STAT3 serine 727 phosphorylation in vivo in growth factor-stimulated cells and also in vitro [37], suggesting a possible dynamic interplay between both MAPK and JAK/STAT3 pathway upon activation by PAP1.

In summary, the present study demonstrates that part of the physiological effects of PAP1, including its self-induction, is strongly dependent on the activation of MAPK signaling pathway (Fig. 7). These results make a contribution to the in-depth knowledge of the mechanisms involved in PAP1 physiological functions.

Fig. 7 Schematic diagram of MAPKs signal transduction pathway induced by PAP1 in AR42J cells. We provide evidence for activation of three MAPKs by PAP1: p38, p44/42, and JNK MAPKs are induced as consequence of the interaction of PAP1 with its receptor and subsequently phosphorylated. Several nuclear factors related to the MAPK family eventually bind the promoter region of PAP1
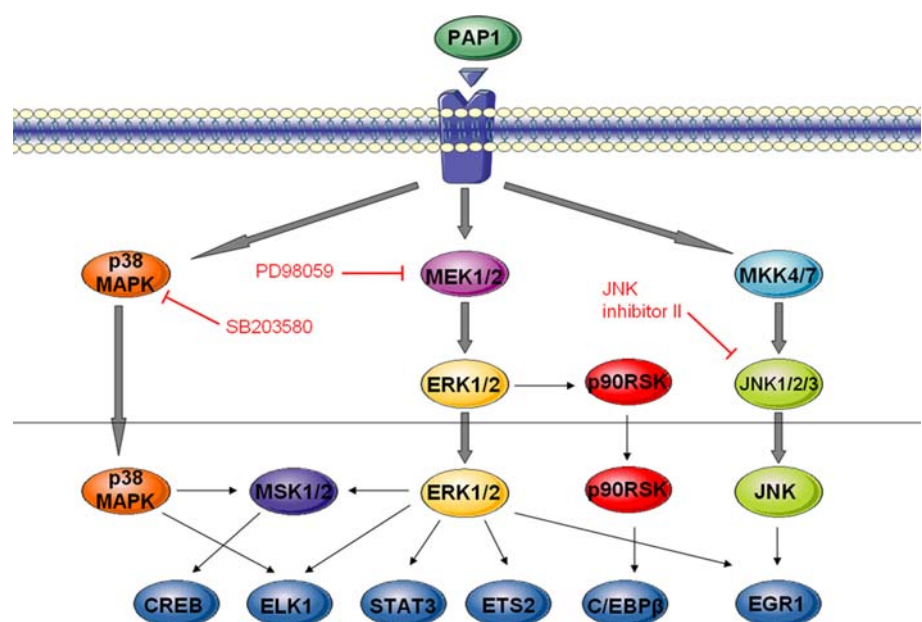
References

1. Keim V, Rohr G, Stockert HG, Haberich FJ (1984) An additional secretory protein in the rat pancreas. Digestion 29:242–249

2. Gironella M, Iovanna JL, Sans M, Gil F, Peñalva M, Closa D, Miquel R, Piqué JM, Panés J (2005) Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease. Gut 54:1244–1253

3. Duplan L, Michel B, Boucraut J, Barthellémy S, Desplat-Jego S, Marin V, Gambarelli D, Bernard D, Berthézène P, Alescio-Lautier B, Verdier JM (2001) Lithostathine and pancreatitis-associated protein are involved in the very early stages of Alzheimer's disease. Neurobiol Aging 22:79–88

4. Christa L, Simon MT, Brezault-Bonnet C, Bonte E, Carnot F, Zylberberg H, Franco D, Capron F, Roskams T, Bréchot C (1999) Hepatocarcinoma-intestine-pancreas/pancreatic associated protein (HIP/PAP) is expressed and secreted by proliferating ductules as well as by hepatocarcinoma and cholangiocarcinoma cells. Am J Pathol 155:1525–1533

5. Xie MJ, Motoo Y, Iovanna JL, Su SB, Ohtsubo K, Matsubara F, Sawabu N (2003) Overexpression of pancreatitis-associated protein (PAP) in human pancreatic ductal adenocarcinoma. Dig Dis Sci 48:459–464

6. Motoo Y, Itoh T, Su SB, Nakatani MT, Watanabe H, Okai T, Sawabu N (1999) Serum levels of pancreatitis-associated protein in digestive diseases with special reference to gastrointestinal cancers. Dig Dis Sci 44:1142–1147

7. Demaugre F, Philippe Y, Sar S, Pileire B, Christa L, Lasserre C, Brechot C (2004) HIP/PAP, a C-type lectin overexpressed in hepatocellular carcinoma, binds the RII alpha regulatory subunit of cAMP-dependent protein kinase and alters the cAMP-dependent protein kinase signalling. Eur J Biochem 271:3812–3820

8. Dusetti NJ, Mallo GV, Ortiz EM, Keim V, Dagorn JC, Iovanna JL (1996) Induction of lithostathine/reg mRNA expression by serum from rats with acute pancreatitis and cytokines in pancreatic acinar AR-42J cells. Arch Biochem Biophys 330:129–132

9. Bimmler D, Schiesser M, Perren A, Scheele G, Angst E, Meili S, Ammann R, Graf R (2004) Coordinate regulation of PSP/reg and PAP isoforms as a family of secretory stress proteins in an animal model of chronic pancreatitis. J Surg Res 15:122–135

10. Graf R, Schiesser M, Lüssi A, Went P, Scheele GA, Bimmler D (2002) Coordinate regulation of secretory stress proteins (PSP/reg, PAP I, PAP II, and PAP III) in the rat exocrine pancreas during experimental acute pancreatitis. J Surg Res 105:136–144

11. Bartoli C, Baeza N, Figarella C, Pellegrini I, Figarella-Branger D (1998) Expression of peptide-23/pancreatitis-associated protein and Reg genes in human pituitary and adenomas: comparison with other fetal and adult human tissues. J Clin Endocrinol Metab 83:4041–4046

12. Nishimune H, Vasseur S, Wiese S, Birling MC, Holtmann B, Sendtner M, Iovanna JL, Henderson CE (2000) Reg-2 is a motoneuron neurotrophic factor and a signaling intermediate in the CNTF survival pathway. Nat Cell Biol 2:906–914

13. Ortiz EM, Dusetti NJ, Vasseur S, Malka D, Bödeker H, Dagorn JC, Iovanna JL (1998) The pancreatitis-associated protein is induced by free radicals in AR42J cells and confers cell resistance to apoptosis. Gastroenterology 114:808–816

14. Malka D, Vasseur S, Bödeker H, Ortiz EM, Dusetti NJ, Verrando P, Dagorn JC, Iovanna JL (2000) Tumor necrosis factor alpha triggers antiapoptotic mechanisms in rat pancreatic cells through pancreatitis-associated protein I activation. Gastroenterology 119:816–828

15. Simon MT, Pauloin A, Normand G, Lieu HT, Mouly H, Pivert G, Carnot F, Tralhao JG, Brechot C, Christa L (2003) HIP/PAP stimulates liver regeneration after partial hepatectomy and combines mitogenic and anti-apoptotic functions through the PKA signaling pathway. FASEB J 17:1441–1450

16. Livesey FJ, O'Brien JA, Li M, Smith AG, Murphy LJ, Hunt SP (1997) A Schwann cell mitogen accompanying regeneration of motor neurons. Nature 390:614–618

17. Moucadel V, Soubeyran P, Vasseur S, Dusetti NJ, Dagorn JC, Iovanna JL (2001) Cdx1 promotes cellular growth of epithelial intestinal cells through induction of the secretory protein PAP I. Eur J Cell Biol 80:156–163

18. Christa L, Carnot F, Simon MT, Levavasseur F, Stinnakre MG, Lasserre C, Thepot D, Clement B, Devinoy E, Brechot C (1996) HIP/PAP is an adhesive protein expressed in hepatocarcinoma, normal Paneth, and pancreatic cells. Am J Physiol 271:G993–G1002

19. Valery C, Vasseur S, Sabatier F, Iovanna JL, Dagorn JC, Grob JJ, Verrando P (2001) Pancreatitis associated protein I (PAP-I) alters adhesion and motility of human melanocytes and melanoma cells. J Invest Dermatol 116:426–433

20. Vasseur S, Folch-Puy E, Hlouschek V, Garcia S, Fiedler F, Lerch MM, Dagorn JC, Closa D, Iovanna JL (2004) p8 improves pancreatic response to acute pancreatitis by enhancing the expression of the anti-inflammatory protein pancreatitis-associated protein I. J Biol Chem 279:7199–7207

21. Zhang H, Kandil E, Lin YY, Levi G, Zenilman ME (2004) Targeted inhibition of gene expression of pancreatitis-associated proteins exacerbates the severity of acute pancreatitis in rats. Scand J Gastroenterol 39:870–881

22. Gironella M, Folch-Puy E, LeGoffic A, Garcia S, Christa L, Smith A, Tebar L, Hunt SP, Bayne R, Smith AJ, Dagorn JC, Closa D, Iovanna JL (2007) Experimental acute pancreatitis in PAP/HIP knock-out mice. Gut 56:1091–1097

23. Folch-Puy E, Granell S, Dagorn JC, Iovanna JL, Closa D (2006) Pancreatitis-associated protein I suppresses NF-kappa B activation through a JAK/STAT-mediated mechanism in epithelial cells. J Immunol 176:3774–3779

24. Sandoval J, Rodríguez JL, Tur G, Serviddio G, Pereda J, Boukaba A, Sastre J, Torres L, Franco L, López-Rodas G (2004) RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. Nucleic Acids Res 32:e88

25. Rodríguez JL, Sandoval J, Serviddio G, Sastre J, Morante M, Perrelli MG, Martínez-Chantar ML, Viña J, Viña JR, Mato JM, Avila MA, Franco L, López-Rodas G (2006) Id2 leaves the chromatin of the E2F4-p130-controlled c-myc promoter during hepatocyte priming for liver regeneration. Biochem J 398:431–437

26. Dusetti NJ, Ortiz EM, Mallo GV, Dagorn JC, Iovanna JL (1995) Pancreatitis-associated protein I (PAP I), an acute phase protein induced by cytokines. Identification of two functional interleukin-6 response elements in the rat PAP I promoter region. J Biol Chem 270:22417–22421

27. Seed B, Sheen JY (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene 67:271–277

28. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005)

MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21:2933–2942

29. Viterbo D, Bluth MH, Lin YY, Mueller CM, Wadgaonkar R, Zenilman ME (2008) Pancreatitis-associated protein 2 modulates inflammatory responses in macrophages. J Immunol 181:1948–1958

30. Krishna M, Narang H (2008) The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell Mol Life Sci 65:3244–3525

31. Kondoh K, Torii S, Nishida E (2005) Control of MAP kinase signaling to the nucleus. Chromosoma 114:86–91

32. Lieu HT, Simon MT, Nguyen-Khoa T, Kebede M, Cortes A, Tebar L, Smith AJ, Bayne R, Hunt SP, Bréchot C, Christa L (2006) Reg2 inactivation increases sensitivity to Fas hepatotoxicity and delays liver regeneration post-hepatectomy in mice. Hepatology 44:1452–1464

33. Pereda J, Sabater L, Cassinello N, Gómez-Cambronero L, Closa D, Folch-Puy E, Aparisi L, Calvete J, Cerdá M, Lledó S, Viña J, Sastre J (2004) Effect of simultaneous inhibition of TNF-alpha production and xanthine oxidase in experimental acute pancreatitis: the role of mitogen activated protein kinases. Ann Surg 240:108–116

34. Dabrowski A, Grady T, Logsdon CD, Williams JA (1996) Jun kinases are rapidly activated by cholecystokinin in rat pancreas both in vitro and in vivo. J Biol Chem 271:5686–5690

35. Schafer C, Ross SE, Bragado MJ, Groblewski GE, Ernst SA, Williams JA (1998) A role for the p38 mitogen-activated protein kinase/Hsp 27 pathway in cholecystokinin-induced in the actin cytoskeleton in rat pancreatic acini. J Biol Chem 273:24173–24180

36. Wagner AC, Metzler W, Hofken T, Weber H, Göke B (1999) p38 map kinase is expressed in the pancreas and is immediately activated following cerulein hyperstimulation. Digestion 60:41–47

37. Chung J, Uchida E, Grammer TC, Blenis J (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 17:6508–6516