PMA up-regulates the transcription of Axl by AP-1 transcription factor binding to TRE sequences via the MAPK cascade in leukaemia cells

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Background. Axl is a receptor tyrosine kinase promoting anti-apoptosis, invasion and mitogenesis, and is highly expressed in different solid cancers. Axl basal transcriptional activity is driven by Sp1/Sp3, and overexpression of MZF-1 (myeloid zinc-finger 1) induces Axl transcription and gene expression. Furthermore, Axl expression is epigenetically controlled by CpG hypermethylation; however, little is known about inducible Axl gene expression and Axl regulation in haematopoetic malignancies.

Results. In the present study, we studied Axl transcriptional regulation under PMA-stimulated conditions in leukaemia cells. Luciferase analysis with sequential 5′-deletion constructs revealed that the −660/−580 region of the Axl promoter is indispensable for induced promoter activity under PMA stimulation. This region includes AP-1 (activator protein 1)/CREB [CRE (cAMP-response-element)-binding protein] motifs, five times partially overlapping TGCGTG repeats and multiple GT repeats. Mutational, supershift and ChIP (chromatin immunoprecipitation) analysis determined that AP-1 family members bind to AP-1 motifs and to the 5×TGCGTG overlapping repeats, thus transactivating Axl promoter activity. Furthermore, specific inhibitors of PKC (protein kinase C), ERK1/2 (extracellular-signal-regulated kinase 1/2) and p38 reduced Axl expression. Additionally, mithramycin treatment abolished constitutive and PMA-induced Axl expression.

Conclusions. Taken together the results of the present study suggest that PMA-induced Axl gene expression in leukaemia cells is mediated by AP-1 motifs and 5×TGCGTG repeats within the promoter region −660/−580, and through the PKC/ERK1/2/AP-1 or PKC/p-38/AP-1 signalling axis.

Introduction

Axl receptor tyrosine kinase belongs to the Tyro3 family, originally identified as a transforming gene in human leukaemias (Janssen et al., 1991; O’Bryan et al., 1991). The human Axl protein has a molecular mass of 140 kDa, and an approximately equal distribution of amino acids on either side of the plasma membrane with two immunoglobulin-like domains and two fibronectin-type III domains in the extracellular region, and a distinctive intracellular kinase domain (O’Bryan et al., 1991; Graham et al., 1994; Lai et al., 1994). Gas6 is a known ligand for Axl, and the Gas6–Axl axis is known to transduce anti-apoptotic signals (Demarchi et al., 2001). Axl is not
only expressed as a transmembrane protein, but is also cleaved at its extracellular domain to produce a sAxl (soluble Axl) form of ~65 kDa. In mouse cells, it has been shown that sAxl generation is mediated by ADAM10 (a disintegrin and metalloproteinase 10) proteolysis, and high levels of sAxl were found as a heterocomplex with the ligand Gas6 in serum (Costa et al., 1996; Budagian et al., 2005a). Overexpression of Axl can transform fibroblasts, even in the absence of Gas6 (Burchert et al., 1998) and is associated with invasiveness and metastasis in various cancer types. Axl overexpression was also detected in peritoneal metastatic nodules of colon cancers, prostate carcinoma, gastric sarcoma, certain types of breast cancers, myeloid leukaemia and some other cancer types (Neubauer et al., 1994; Weiner et al., 1994; Craven et al., 1995; Jacob et al., 1999; Meric et al., 2002; Wu et al., 2002; Sun et al., 2003).

Signals transmitted through PKC (protein kinase C) can potentially activate the network of MAPK (mitogen-activated protein kinase), which plays an important role in regulating the response of cells to various extracellular stimuli, such as PMA (Rubinfeld and Seger, 2004; Kolch et al., 2005). The MAPK signalling pathways are activated by sequential phosphorylation steps, through five pathways mediated by JNK (c-Jun N-terminal kinase), ERK (extracellular-signal-regulated kinase) 1/2, p38 MAPK, ERK5 and ERK3/4. Activated MAPK kinase pathways activate AP-1 (activator protein 1), which is a heterodimeric complex of structurally and functionally related members of the Jun (c-Jun, JunB or JunD) and Fos (c-Fos, FosB, Fra-1 or Fra-2) family. Additionally, some members of the ATF (activating transcription factor) (ATF4, ATF-2 and ATF-3) and JDP (J-domain-containing protein) (JDP-1 and JDP-2) subfamilies, which share structural similarities and form heterodimeric complexes with AP-1 proteins (predominantly with Jun proteins), can bind to TRE (PMA-response element)-like sequences (5’-TGAC/GTCA-3’), as does AP-1. The net activity of AP-1 can be regulated by changes in the transcription of genes encoding AP-1 subunits, by controlling the stability of their mRNAs, by post-translational processing and turnover of pre-existing or newly synthesized AP-1 subunits and by specific interactions between AP-1 proteins and other transcription factors and cofactors. The activation of AP-1 is involved in cellular proliferation, apoptosis, differentiation and carcinogenesis (Angel and Karin, 1991; Shaulian and Karin, 2002, Eferl and Wagner, 2003).

Our previous studies (Mudduluru and Allgayer, 2008; Mudduluru et al., 2010) have shown that constitutive expression of Axl in solid cancer is driven by Sp1/Sp3, and that overexpression of MZF-1 (myeloid zinc finger 1) transactivates Axl transcription and gene expression. Moreover, Axl and MZF-1 are co-expressed in colorectal cancer tissues. Furthermore, Axl expression is epigenetically controlled by CpG hypermethylation. Still, the transcriptional regulation of Axl in blood cells and under stimulated conditions is not clearly known. Therefore, the present study was performed with leukaemia cells as a model system, and with PMA as an oncogenic stimulus to investigate the essential promoter region of Axl, its transcriptional regulation and inducing signalling molecules under stimulated conditions.

Results

**PMA (5 nM) induces Axl gene expression in leukaemia cells**

To determine the effect of PMA on Axl expression, K562 and HL-60 cells were stimulated with PMA in a dose-dependent manner for 24 h. Endogenous Axl mRNA amounts were screened by quantitative-PCR analysis. Both cell lines showed inducible Axl gene expression in a dose-dependent manner (Figures 1a and 1b). We determined that 5 nM PMA is sufficient for stimulation of K562 and HL-60 cells, in which a 2-fold (K562) and 10-fold (HL-60) increase in Axl gene expression is shown (Figures 1a and 1b). Furthermore, 5 nM PMA stimulation significantly induced Axl protein amounts in both cell lines (Figures 1b and 1d). Therefore 5 nM PMA was used for stimulation in all further experiments.

**727/−556 bp of the Axl promoter region is mandatory for PMA-induced promoter activity**

To determine the minimal Axl promoter region required for PMA stimulation, we transfected a series of deletion luciferase constructs into K562 cells. The AxlP1 construct (−1276/+7) exhibited a maximum luciferase activity in both stimulated and unstimulated conditions as compared with pGL3-Basic (Figure 2a). Further deletion constructs, AxlP2 (−1010/+7), AxlP3 (−727/+7), AxlP4 (−556/+7) and AxlP5 (−478/+7) showed
Figure 1 | PMA induces Axl gene expression
(a and c) K562 and HL-60 cells were stimulated with different concentrations of PMA for 24 h along with DMSO controls, and Axl mRNA expression was determined by real-time PCR. GAPDH served as an internal control. (b and d) Both of the cell lines were treated with 5 nM PMA or DMSO, or neither of them, as indicated. Cell lysates were prepared and Axl was detected by Western blot analysis. Untreated or DMSO-treated cells served as controls and β-actin served as an internal loading control. TPA, PMA.

a gradual decrease in luciferase activity in both unstimulated as well as PMA-stimulated conditions, and AxlP4 completely lost the ability to be stimulated by PMA. AxlD1 (−2376/−556) and AxlD2 (−2376/−770) lack the core promoter and, additionally, AxlD2 lacks a region spanning −770/−556, this construct showing almost no luciferase activity, comparable with the pGL3-Basic vector activity (Figure 2a) in both stimulated and unstimulated conditions. The −2376/+7 reporter construct did not show any further significant induction in both conditions when compared with the AxlP1 (−1276/+7) construct (results not shown), indicating that the main promoter activity for PMA stimulation lies within the −1276/+7 bp region, this being comparable with the results seen for constitutive promoter activity in Rko, HCT116 and HeLa cells (Mud-duluru and Allgayer, 2008). From all of the deletion experiments, we conclude that these data suggest that the 5′-flanking region located between −727 and −556 bp is required for PMA-stimulated Axl promoter activity in leukaemia cells. Correspondingly, a TRANSFAC database search and manual screening predicted AP-1/CREB [CRE (cAMP-response-element)-binding protein]-binding motifs between −545 and −530 bp, five overlapping TGCGTG motifs between −628 and −606 bp, and a GT-rich sequence (−610 to −589) in this region of the Axl promoter (Figure 2b). Apart from these, one more AP-1 consensus sequence was predicted at −1030/−1024 bp of the Axl promoter.

AP-1 and 5 × TGCGTG repeats mediate PMA-induced Axl promoter activity
To analyse the functional relevance of predicted motifs in this important region of the promoter (−727/−556 bp), AP-1 putative motifs were mutated at four/five bases by site-directed
mutagenesis within the AxlP1 (−1276 to +7) reporter plasmid. Different mutated constructs were generated, with mutations either in single regions (AP-1 I, AP-1 II, AP-1 III and AP-1 IV) or all of them (AP-1- I, II, III, IV) (Figures 2c and 2d). From our previous results, it was shown that the Axl core promoter is driven by Sp1 and Sp3 in colorectal cancer cell lines (Mudduluru and Allgayer, 2008). Therefore, to study the additional relevance of Sp family members under stimulated conditions, we also used constructs mutated with Sp-binding domains of the Axl promoter (Sp1 abcd e) for the present study (Mudduluru and Allgayer, 2008). Mutated and wild-type constructs were transfected into the K562 and HL-60 cells and compared for reporter activity. We observed that all mutants for single AP-1 motifs reduced the PMA-stimulated promoter activity as compared with the wild-type AxlP1. Especially the AP-1 II and AP-1 III mutant constructs significantly reduced the PMA-stimulated promoter activity ($P < 0.05$), whereas AP-1 IV showed the trend ($P = 0.058$) in K562 cells. In HL-60 cells, the AP-1 III mutant construct showed significant reduction in the promoter activity ($P = 0.021$), and others showed reduction in the promoter activity. The construct where there was mutation of the four AP-1 motifs significantly reduced the PMA-stimulated promoter activity in both of the cell lines ($P = 0.0007$ and $P = 0.018$ respectively). Unstimulated promoter activity was not significantly altered with any of the AP-1
mutations (Figure 2c). This confirms that all four AP-1 motifs mediate PMA stimulation of the promoter, especially AP-1 II and III. However, they do not affect basal promoter activity as compared with the Sp family binding motifs (Sp1 abcde; Figure 2c). In addition, the Sp1 abcde mutant construct did not show any significant increased promoter activity upon PMA stimulation (Figure 2c). EMSA (electrophoretic mobility-shift assay) and Western blot analysis for Sp-binding motifs, and expression of Sp1 and Sp3 after PMA stimulation did not show an increase in binding efficiency and expression of Sp1 and Sp3 (Supplementary Figures S1a and S1b at http://www.biolcell.org/boc/103/boc1030021add.htm). This clearly implicates that the Sp-binding motifs are the essential mediators of basic promoter activity, whereas the AP-1 motifs are mediators of PMA-inducible promoter activity in leukaemia cells.

PMA increases the abundance of AP-1 family members

Functional and mutagenesis studies revealed that AP-1 motifs are important for PMA-stimulated Axl promoter activity. To investigate whether this PMA-induced promoter activity is due to an increase of expression or stabilization of AP-1 transcription factors, sequential Western blot analysis was performed for AP-1 and Axl proteins. Protein amounts of all AP-1 members were increased in a time-dependent manner, among them c-Fos being the one with an increase after 1 h of stimulation. Starting at 2 h, Fos B protein increased, with a maximum level at 4 h. After 30 min of stimulation, activated c-Jun was observed, and this was constantly present for 24 h. In addition, total protein levels of c-Jun were increased, starting at 2 h. Axl protein levels were increased after 4 h of treatment and were further increased at 24 h (Figure 3a). Most of the AP-1 family members’ gene expression was induced by PMA-stimulation in K562 cells. Activated c-Jun levels were stable from 8 to 24 h after stimulation, which was paralleled by an increase of Axl protein levels at 8 h and a further 4-fold increase of Axl protein at 24 h. Taken together, these data show that PMA activates c-Jun and stabilizes the expression of all AP-1 family members, which is followed by an increase of Axl protein expression in K562 cells. Since we observed a higher activation/expression of AP-1 transcription factors at 8 h post-stimulation with PMA, we used this time point for EMSA and supershift analysis to show the binding of these transcription factors to the respective cis-elements in the Axl promoter.

AP-1 family nuclear proteins bind to the Axl promoter

To demonstrate that PMA-induced Axl gene expression is dependent on AP-1 family transcription factors binding to the promoter, gel-shift and supershift analysis was performed using nuclear extracts either with DMSO- or PMA-stimulated K562 cells. EMSA and supershift analysis was performed with oligonucleotides corresponding to the AP-1-binding motifs (Figure 2d) and the GT-rich region. To determine specific binding of AP-1 to these motifs, a 50-fold excess of unlabelled wild-type, or a 50-fold excess of an oligonucleotide mutated for the respective AP-1-binding motif (Supplementary Figure S2 at http://www.biolcell.org/boc/103/boc1030021add.htm) were used for cold (non-radioactive) competition. As shown in Supplementary Figure S2, the competition with wild-type oligonucleotides completely abolished specific binding of transcription factors, whereas, with the mutated oligonucleotides as competitors, the formation of specific complexes stayed intact, demonstrating binding specificity for these motifs.

To investigate the binding of specific AP-1 family members to these motifs, a comparative supershift analysis with all AP-1 family members was performed with nuclear extracts of K562 cells, with or without PMA stimulation (Figure 2d). PMA stimulation increased the abundance of specific complexes to the AP-1 motifs. Incubation of AP-1 family antibodies identified phospho-c-Jun within the AP-1 complexes at all four AP-1 motifs and at the GT-rich region, under PMA-unstimulated conditions (Figures 3b and 3c, and Supplementary Figures S3a and S3b, top panel at http://www.biolcell.org/boc/103/boc1030021add.htm). Following PMA stimulation, Fos B and Fra 1 were identified to be increased within the AP-1 complexes at all sites, and also at the GT-rich region (Figures 3b and 3c, and Supplementary Figures S3a and S3b, bottom panel). Additionally, Jun B was identified within complexes bound to the AP-1 I and AP-1 IV motif, and Jun B, Jun D and c-Fos within complexes at the AP-1 II and AP-1 III motif (Figures 3b and
Figure 3 | AP-1 family members bind to the Axl promoter, and their abundance increases after PMA stimulation
(a and b) Supershift analysis was performed with (bottom panel) or without (top panel) PMA stimulation (for 8 h) and nuclear extracts of K562, for AP-1 family members (c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra 1 and Fra 2) using AP-1 I (−628/−606 bp) and AP-1 II and III (−655/−640 bp) as radioactive probes. IgG served as the negative control. Specific supershifted complexes are indicated with arrows. (c) K562 cells were stimulated with 5 nM PMA for the time points indicated, followed by preparation of total cell lysates. Untreated cells were used as a control. Phospho-c-Jun, c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra 1, Fra 2 and Axl protein levels were determined by Western blot analysis. β-Actin served as the internal control. The results shown are representative of two similar experiments. (d) The in vivo association of phospho-c-Jun with the Axl promoter was evaluated with a ChIP assay in K562 and HL-60 cells after 24 h of DMSO, PMA, Gö6983, or Gö6983 with PMA treatment. Immunoprecipitated DNA with the specific antibody was amplified by real-time PCR (SYBR® Green) using specific primers. TPA, PMA.

3c, and Supplementary Figures S3a and S3b, bottom panel).

The AP-1 II and AP-1 III motifs harbour CRE and TRE consensus sequences, which could bind CREB and AP-1 transcription factors respectively. To define binding of phospho-CREB and phospho-c-Jun transcription factors together at these motifs, supershift analysis was performed. No significant differences in band intensities were observed between unstimulated and PMA-stimulated supershift bands for CREB and phospho-CREB; however, an intense shifted band was observed with phospho-c-Jun following PMA stimulation (Supplementary Figure S4b at http://www.biolcell.org/boc/103/boc1030021add.htm), even though increased activated p-CREB levels were observed within nuclear proteins (Supplementary Figure S4a). These results suggest that AP-1 family transcription factors are binding preferentially to the
specific AP-1 motifs in the Axl promoter, as opposed to CREB transcription factors.

ChIP (chromatin immunoprecipitation) analysis was performed to determine the in vivo relevance of AP-1 transcription factor binding to the Axl promoter, comparing DMSO treatment with PMA stimulation, and, to get initial insights into putative signalling mediators, with a PKC/PKC inhibitor in K562 and HL-60 cells. A non-specific region of the uPAR [uPA (urokinase-type plasminogen activator) receptor] promoter was used as a negative control (Mudduluru and Allgayer, 2008), which did not show any specific changes after treatment (results not shown). PMA stimulation increased the binding of phospho-c-Jun to the Axl promoter; also, 1 h pre-treatment with Gö6983 decreased the binding of phospho-c-Jun (Figure 3d). Similar results were observed in Western blot analysis; Gö6983 inhibited c-Jun activation (Figure 4b). These data support the notion that, in vivo, AP-1 binds to the predicted AP-1 consensus and GT-rich region of the Axl promoter upon PMA-stimulation, and that the PKC pathway might be one important mediator.

The PKC/MAPK signalling pathway mediates PMA-induced Axl gene expression

To investigate signalling molecules mediating PMA-induced Axl gene expression, luciferase assay, EMSA, quantitative PCR and Western blot analysis were performed following treatment of the cells with various chemical inhibitors of the MAPK signalling pathway prior to PMA stimulation. K562 and HL-60 cells were transfected with AxlP1 reporter constructs and treated with the specific inhibitors for 45 min and stimulated with PMA for 24 h (Figure 5a). PKC and ERK1/2 inhibitors significantly reduced the Axl promoter activity, whereas a p38 inhibitor showed the trend and a JNK inhibitor showed reduced promoter activity. Similar results were observed with EMSA analysis with inhibitor treatment (Figure 5b). PKC, ERK1/2 and p38 inhibitors showed reduced PMA-induced AP-1 binding to the Axl promoter. Additionally, K562 or HL-60 cells were untreated, treated with DMSO or treated with PMA following 45 min of pretreatment with PKC/JNK/ERK/p38 inhibitors, or the GC-rich binding compound mithramycin (Figure 4). No differences in the activation and expression of PKC signalling molecules and in Axl expression and protein amounts were observed between untreated and DMSO-treated samples. PMA-induced Axl gene expression was completely abolished by the PKC inhibitor Gö6983 in parallel with the downstream signalling molecules ERK, p38, JNK and phospho-c-Jun, in both of the cell lines (Figures 4a and 4b). Specific inhibitors of ERK (U0216), p38 (SB202190) and JNK (SP600125) partially abolished PMA-induced Axl gene expression in K562 cells (Figure 4a). Both the ERK1/2 and p38 inhibitors completely abolished Axl gene expression in the HL-60 cell line (Figure 4a). Furthermore, Western blot analysis showed similar results in K562 cells (Figures 4c and 4d). Individual inhibitors of JNK/ERK/p38 could not abolish the activation of c-Jun completely, as indicated by Western blotting for phosphorylated c-Jun (Figures 4c and 4d). This could be due to the leakage of signalling through other MAPK signalling molecules. This suggests that PMA-induced signalling might be working mainly through ERK and p38. However, the differences in PMA-induced Axl gene expression through MAPK signalling in these cell lines need to be further investigated thoroughly. Apart from the findings presented, post-transcriptional regulation of Axl cannot be ruled out after PMA stimulation. Furthermore, to gain additional information on the PMA-induced effect on basal Axl gene expression, mithramycin, which has already been shown to abolish Axl basal gene expression via the prevention of Sp binding in colorectal cell lines (Mudduluru and Allgayer, 2008), was included. PMA-activated MAPK could not induce Axl gene expression after mithramycin treatment (Figure 4c), even though the MAPK signalling cascade was active, suggesting that basal Axl gene expression via Sp factors is a prerequisite for inducible expression. Taken together, these results suggest that PMA-induced Axl gene expression is mainly regulated via PKC and MAPK/ERK1/2 and MAPK/p38 pathways, and it further reconfirms in leukaemia cells that Sp family transcription factors are required for constitutive Axl gene expression.

Discussion

Axl has been discussed as a transforming gene in leukaemia (Janssen et al., 1991; O’Bryan et al., 1991); however, mechanisms inducing its expression upon oncogenic stimuli in leukaemia are still the subject of investigation. In the present study
we show that, in leukaemia cells, PMA-induced Axl gene expression is mediated by AP-1 family transcription factors bound to AP-1 cis-acting elements within −660 and −580 bp of the promoter region. This region is essential for PMA-induced Axl promoter activity. PMA-stimulation activates AP-1 family members and induces the expression/stabilization of AP-1 family transcription factors that lead to induced Axl gene expression in leukaemia cell lines. Moreover, we found that PMA-stimulated Axl gene expression is mediated through ERK/JNK/p38 signalling.
Figure 5 | The MAPK pathway inhibitors reduce Axl promoter activity and AP-1 binding to its promoter

(a) Luciferase analysis of AxlP1 either with or without pre-treatment of specific inhibitors in K562 and HL-60 cells. (b) EMSA analysis was performed with AP-1 II and III (−655/−640 bp) motif with or without PMA stimulation (8 h) after 45 min of pre-treatment with the specific inhibitors in K562 and HL-60 cells. TPA, PMA.

AP-1 consists of various dimers of the Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun (c-Jun, JunB and JunD), and CREB/ATF protein families, as well as other bZip proteins. In addition, associations have been observed between Fos or Jun and the p65 subunit of NF-κB (nuclear factor κB) (Stein et al., 1993) and ATF-2 and p50-NF-κB (Du et al., 1993). AP-1 transcription factor heterodimers or homodimers bind to a consensus cis-element in the promoters of a number of genes (Adler et al., 1994). Evidence for the critical importance of AP-1 activity in malignant transformation induced by tumour promoters or by oncogenes has been reported (Bernstein and Colburn, 1989; Domann et al., 1994; Dong et al., 1994). The regulation of AP-1 activity is complex and occurs at different levels, including dimer composition, transcriptional and post-translational events, and interaction with ancillary proteins. AP-1 is induced by several external stimuli that increase MAPK activity, inducing activation of c-Fos and c-Jun. PMA, a tumour-promoting agent, often serves as a model agent to study the mechanism by which growth factors regulate growth and differentiation of the cells, and is an endogenous activator of PKC and MAPK pathways (Jaken, 1996; Parekh et al., 2000). AP-1 activation itself can be mediated by AP-1 binding to TRE elements within the promoter, for example of c-Jun and Fra-1, inducing their expression (Angel et al., 1988; Bergers et al., 1995). The −1276/+7 region of the Axl promoter showed maximum activity under PMA-stimulated conditions (Mudduluru and Allgayer, 2008). A TRANSFAC database search, 5' delineation reporter analysis, site-directed mutagenesis, EMSA and supershift analysis of the Axl promoter identified two unique binding motifs of AP-1 and TRE-consensus sequences, which are essential for PMA-stimulated promoter activity (AP-1 II and AP-1 III). Apart from these two, AP-1-binding motifs containing partially overlapping five TGGCGTG repeats, a −607 to −589 bp unique GT-rich motif and a distant −1010 to −1022 bp AP-1 motif show specific binding complexes of AP-1 transcription factors under PMA-stimulated conditions, which are also required for stimulated Axl promoter activity. Axl protein amounts could be detected at 4 h and increased gradually up to 24 h, whereas activated c-Jun was detected at 30 min and achieved maximum activated levels at 12 h. Among AP-1 members, an induction of c-Fos and c-Jun was detected as early as 1 h and 2 h respectively, and achieved maximum
levels 4–12 h after PMA treatment. These findings suggest that c-Jun is being activated at early time points upon PMA treatment and, indeed, transactivates/stabilizes the other AP-1 transcription factors. Thus c-Jun or c-Fos might be playing a major role in the earlier course and the rest of the AP-1 factors may play a major role in the later course of the induction of Axl gene transcription by PMA, a phenomenon which has been described previously, e.g. for Fra1 and proenkephalin (Adiseshaiah et al., 2003; Won et al., 1998). We can speculate that upon PMA stimulation, AP-1 transcription factors are activated and autoregulated or stabilized at their protein levels, and then transactivate Axl gene expression.

c-Jun transcriptionally regulates EGFR [EGF (epidermal growth factor) receptor] and HB-EGF (heparin-binding EGF-like growth factor), thereby controlling keratinocyte proliferation and skin tumour formation (Zenz et al., 2003). Hu et al. (1994) and Kustikova et al. (1998) reported that AP-1 transcription factors stimulate the transcription of MMP (matrix metalloproteinase) 9, MMP1, uPA, uPAR, HMGI(Y) [high-mobility group I (Y)] and E-cadherin, which are tumour progression- and metastasis-associated genes. Since Axl overexpression is associated with increased invasion, metastasis and angiogenesis in various cancer cells, and also its overexpression is reported in different cancers, such as metastatic colon and prostate carcinoma, gastric, endometrial, certain types of breast cancers and sarcomas (Neubauer et al., 1994; Weiner et al., 1994; Craven et al., 1995; Jacob et al., 1999; Meric et al., 2002; Wu et al., 2002; Sun et al., 2003; Budagian et al., 2005b; Shieh et al., 2005; Vajkoczy et al., 2006), the transactivation of Axl by AP-1 following PMA stimulation adds to the list of essential AP-1 target genes in the context of carcinogenesis, invasion, metastasis and angiogenesis.

Signals transmitted through PKC can potentially activate the network of MAPK signal-transduction pathways (ERK1/2, JNK, p38, ERK5 and ERK3/4), which play an important role in regulating the response of cells to various extracellular stimuli, such as PMA (Kast et al., 2003; Rubinfeld and Seger, 2004; Kolch et al., 2005). The conventional position of c-Jun/AP-1 in signal transduction cascades has been downstream of MAPK pathways. Thus we studied the effect of inhibitors of each MAPK pathway on PMA-dependent Axl gene expression. PMA-activated MAPK, activated c-Jun and promoted Axl promoter activity and gene expression (Figure 4), indicating that activation of the MAPK cascade is relevant for PMA-induced Axl gene expression (Angel and Karin, 1991; Minden and Karin, 1997; Kast et al., 2003). Diverse inhibitors of different molecules of MAPK-associated pathways [JNK (SP600125), ERK1/2 (U0126), and p38 (SB202190)] repressed c-Jun activation and reduced AP-1-binding complexes to the Axl promoter, indicating three different lines of signalling in this particular situation (Pulverer et al., 1991; Papavassiliou et al., 1995; Shin et al., 2002; Kast et al., 2003). These findings suggest that inhibition of PKC or downstream signalling molecules of PKC (ERK1/2, JNK, p38) activity results in an inhibition of AP-1 transactivation, which is an important mechanism for Axl transactivation and gene expression under tumorigenic PMA stimulation, suggesting therapeutic considerations for leukaemia. Mithramycin treatment completely blocked PMA-induced Axl gene expression, even though the MAPK signalling cascade was active, which is a confirmation that, also in leukaemia, Sp1/Sp3 is essential for the basal transcriptional activation of Axl gene expression, which is a prerequisite for inducibility (Mudduluru and Allgayer, 2008).

In summary, we have identified an AP-1 family binding region − 660 to −580 bp within the Axl promoter that is indispensable for PMA-induced Axl gene expression in leukaemia cells. Induction of transcription factor AP-1 binding is mainly mediated through the ERK1/2/p38 pathway, and phorbol esters transactivate and/or stabilize the expression of all AP-1 family transcription factors in leukaemia cells.

Materials and methods

Cell culture, reagents and treatments

K562 and HL-60 cells were obtained from the A.T.C.C. and grown at 37°C in RPMI medium supplemented with 10% FCS (fetal calf serum). Mock-treated control cells were handled identically with PMA (Sigma)-stimulated cells with the exception that only medium was added. For inhibition studies, cells were treated with or without the following inhibitors for 45 min: 5 μM G66983 (Calbiochem), 10 μM U0216 (Calbiochem), 10 μM SB202190 (Calbiochem), 100 nM mithramycin (Sigma) and 5 or 10 μM SP600125 (Calbiochem) respectively.

Preparation of protein extracts and immunoblotting

Protein lysates were prepared as described previously by Mudduluru et al. (2008). Briefly, for immunoblotting, samples (50 μg/lane) were boiled for 5 min, separated via SDS/PAGE,
and transferred on to PVDF membranes. After transfer, the membranes were blocked with 5% non-fat skimmed milk containing TTBS (TBS-buffered saline, 50 mM Tris/HCl and 150 mM NaCl, pH 8.0) with 0.1% Tween for 3 h at room temperature (21°C) and then probed with primary antibodies against c-Jun (Santa Cruz Biotechnology, sc-45x), phospho-c-Jun (Santa Cruz Biotechnology, sc-822), Jun B (Santa Cruz Biotechnology, sc-46x), Jun D (Santa Cruz Biotechnology, sc-74x), c-Fos (Santa Cruz Biotechnology, sc-52x), Fos B (Santa Cruz Biotechnology, sc-7203x), Fra1 (Santa Cruz Biotechnology, sc-22794x), Fra2 (Santa Cruz Biotechnology, sc-604x), phospho-Jnk (Cell Signaling Technology, cs-9231s), JNK (Cell Signaling Technology, cs 9252), phospho-ERK1/2 (Cell Signaling Technology, cs 9101s), ERK1/2 (Cell Signaling Technology, cs 9102s), phospho-p38 (Cell Signaling Technology, cs 9211s), p38 (Cell Signaling Technology, cs-9212), Axl (Santa Cruz Biotechnology, sc-1096) or β-actin (Santa Cruz Biotechnology, sc-1616) for 2 h at room temperature. After three washes with TTBS, blots were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase. After final washes with TTBS, the membranes were exposed to film after ECL (enhanced chemiluminescence; Amersham Biosciences).

RNA isolation and RT (reverse transcriptase)–PCR
Total RNA was extracted using the RNeasy Midi kit (Qiagen) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed by random hexamer primers using SuperScript II reverse transcriptase (Invitrogen). The single-strand cDNA was amplified by SYBR® Green quantitative PCR using specific primers as described previously (Mudduluru et al., 2010). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The sequences of the sense and antisense primers used for either semi-quantitative, or SYBR® Green quantitative PCR were: GAPDH (sense, 5′-GTCCTTACACCACTGGAGAAG-3′ and antisense, 5′-TACCACGATGCTTCTGGGTGG-3′) and Axl (sense, 5′-GGTGCGCCTGAAAGACAGATGA-3′ and antisense, 5′-CTCAGATACATCCATGCGACT-3′).

Preparation of nuclear extract and EMSA
Cells were either pre-treated with the respective specific inhibitor for 45 min and/or treated for 8 h with PMA (5 nM), then cells were harvested into 50 ml Falcon tubes and washed with ice-cold PBS. Nuclear extraction, protein concentration estimation and EMSAs were essentially performed as described previously (Mudduluru and Allgayer, 2008; Mudduluru et al., 2010). Briefly, 100 ng of annealed double-stranded oligonucleotides, GT-rich (−609/−569), AP-1 I (5 × TGGGTG repeats: −655/−601), AP-1 II and III (−672/−631), and AP-1 IV (−1036/−1008), of the Axl promoter listed in Supplementary Table S1 (at http://www.biolcell.org/boc/103/boc1030021add.htm) were end-labelled with [γ-^32P]dATP using T4 polynucleotide kinase (NEB), and purified with Sephadex G-50 columns (Amersham Bioscience). The DNA-binding reaction was conducted on ice for 20 min with 5 μg of K562 cell nuclear extract. For supershift assays, antibodies were incubated with the reaction mixture at 4°C for an additional 60 min. Samples were analysed on 5% polyacrylamide gel in 0.25 × Tris/borate/EDTA buffer at 10 V/cm for 3.5 h. The gel was dried and analysed by autoradiography.

Generation of luciferase reporter constructs
Luciferase reporter constructs of the Axl promoter generated as described by Mudduluru et al. (2008). Two additional reporter constructs were generated (Mudduluru and Allgayer, 2008), lacking the transcriptional start site and the core promoter, using specific reverse primers. The primer sequences are listed in Supplementary Table S2 (at http://www.biolcell.org/boc/103/boc1030021add.htm). Mutant constructs were generated using the QuikChange® XL site-directed mutagenesis kit from Stratagene using AxlP1 (−1276 to +7) as a template. The sequences of the mutant oligonucleotides are shown in Supplementary Table S1. Successful incorporation of the mutations was confirmed by automated sequencing.

Transfection and luciferase assays
For luciferase assays, 0.5 × 10^6 K562 cells were plated in 24-well plates at the time of transfection. Luciferase reporter plasmids (0.5 μg of each) were transfected with the Effectene® transfection reagent (Qiagen). For AP-1/Sp family binding motif mutation and specific inhibitor-treated luciferase assay analysis, cells were electroporated with the Amaxa Cell Line Nucleofector Kit V (Lonza) according to the manufacturer’s protocol into 2 × 10^6 cells with 2 μg of respective reporter plasmid. For all luciferase reporter assays, 25 ng of pRL-TK (Renilla luciferase-thymidine kinase; Promega) was also co-transfected and measured to normalize transfection efficiency. After 12 h of transfection, cells were either pre-treated with specific inhibitors for 45 min and/or stimulated with 5 nM of PMA. After 24 h, cells were washed twice with PBS and lysed with 100 μl of passive lysis buffer (Promega) for 20 min. Luciferase activity of 20 μl of cell lysate was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Assays for all samples were performed in triplicate, and the results were averaged.

ChIP assay
ChIP assays were performed as described previously (Leupold et al., 2007; Mudduluru and Allgayer, 2008) using either anti-phospho-c-Jun (Santa Cruz Biotechnology, sc-822) X and anti-γ-IgG (as a control; Santa Cruz Biotechnology, sc-2338 X) antibodies. K562 or HL-60 cells were either mock-treated with DMSO or treated with G66983 (5 μM) in the respective samples 45 min prior to PMA (5 nM) stimulation. After 24 h, cells were processed as described previously (Leupold et al., 2007; Mudduluru and Allgayer, 2008). Phospho-c-Jun-binding intensity to the Axl promoter was measured using SYBR® Green PCR with specific primers: sense 5′-AGTGTGTCTGTTGGCCAGAATA-3′ and antisense 5′-CTAGATCCACTTTCAC-3′.

Statistical analysis
Statistical analyses were performed using SPSS statistical software. A Student's t test was performed for data with normal distribution, and the Wilcoxon test for all other analyses. P values of ≤0.05 were considered as statistically significant.
Author contribution

Concept and design of the research was by Giridhar Mudduluru. The experiments were carried out by Giridhar Mudduluru and Jörg Leupold; collection and/or assembly of data was by Giridhar Mudduluru; and data analysis and interpretation was by Giridhar Mudduluru and Philipp Stroebel. The manuscript was written by Giridhar Mudduluru and Heike Allgayer. Financial support for the study was obtained by Heike Allgayer. All authors approved the final manuscript.

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MAPK pathways in Axl gene expression


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Supplementary online data

PMA up-regulates the transcription of Axl by AP-1 transcription factor binding to TRE sequences via the MAPK cascade in leukaemia cells

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Figure S1 | EMSA and Western blot for Sp family members

(a) EMSA analysis of 8 h PMA-stimulated or unstimulated nuclear extracts of K562 cells with Sp-binding motifs (Mudduluru and Allgayer, 2008). (b) Sequential Western blot analysis for Sp1 and Sp3 was performed with PMA-stimulated K562 cells as indicated. TPA, PMA.

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Figure S2 | AP-1 transcription factors bind to putative binding sites of the Axl promoter

EMSA was performed with or without PMA-stimulated nuclear extract of K562 cells and a γ-32P-labelled oligonucleotide probe corresponding to the GT-rich, AP-1 I, AP-1 II and AP-1 III sites of the Axl promoter. For competition, a 50-fold excess of unlabelled wild-type or mutant oligonucleotides was used. The GT-rich region (−590/−610 bp) mutated for T with A, was unable to abolish the binding of PMA-induced complexes [left-hand panel (GT-rich)], yet no specific binding complexes were observed under unstimulated conditions. It shows that under stimulated conditions, PMA-induced transcription factors bind to this GT-rich motif. Gel-shift analysis with AP-1 showed higher binding of specific complexes with PMA-stimulated samples which were not observed in unstimulated conditions. Moreover, base conversion of GC into TT in this AP-1 I (middle panel) site failed to abolish the PMA-induced binding complexes when used as mutant competitor oligonucleotides. These results showed that the complexes bound to AP-1 I motifs are highly specific. Higher binding of specific complexes bound to AP-1 II and III (consisting of TRE and CRE elements in close proximity in the promoter) were observed under PMA-stimulated conditions (right-hand panel). Individual competition with oligonucleotides containing mutations in TRE elements, as well as CRE elements, partially abolished the binding complexes; however, oligonucleotides containing mutations on both TRE and CRE elements failed to do so. TPA, PMA.

<table>
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<tr>
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<th>Stimu</th>
<th>UnSt</th>
<th>Stimu</th>
<th>UnSt</th>
<th>Stimu</th>
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<td>50xN</td>
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<td>50xAP II EM3</td>
<td>50xAP III EM</td>
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</table>

UnSt: Unstimulated; Stimu: Stimulated with TPA (5 nM) for 8 hours. 50xN: 50 times cold competition; EM: EMSA Mutated oligo; M: Mutated oligo
MAPK pathways in Axl gene expression

Figure S3 | Supershift analysis of AP-1 family members
(a and b) Supershift analysis was performed as described in the Materials and methods section of the main text, with (bottom panel) and without (top panel) 8 h PMA-stimulated nuclear extracts of K562, for AP-1 (c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra 1 and Fra 2) using a GT-rich motif (−590/−610 bp) and AP-1 IV (−1013/−1019 bp) as radioactive probes. IgG served as the negative control. Specific supershifted complexes are indicated. (c) Schematic presentation of an important region taken for CHIP analysis. For, forward; Rev, reverse.
Figure S4 | Western blot and supershift analysis for CREB
(a) Western blot analysis for phospho-CREB and total CREB was performed with nuclear extracts from 8 h PMA-stimulated or unstimulated samples. DNA polymerase ε served as an internal control. (b) Supershift analysis of 8 h PMA-stimulated or unstimulated nuclear extracts of K562 cells was performed by adding antibodies for phospho-CREB, CREB, and phospho-c-Jun with AP-1 II and III motifs (−655/−640 bp) as the radioactive probe. IgG served as the negative control. Specific supershifted complexes are indicated. TPA, PMA.
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Table S1 | Primers used for EMSA and supershift analysis
The position is from the upstream translation start site. Lower-case letters represent the sequences used to mutate the respective transcription-factor-binding motif. Letters in italics indicate the oligonucleotides used for EMSA analysis, apart from the respective normal and mutated oligonucleotides.

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Table S2 | Primers used for Axl promoter cloning
The position is from the upstream translation start site.

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Reference

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