BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 4–9 (1997)
ARTICLE NO. RC976715

Identification and Characterization of a Novel Monocyte/ Macrophage Differentiation-Dependent Gene That Is Responsive to Lipopolysaccharide, Ceramide, and Lysophosphatidylcholine

Anett Pietzsch, Christa Büchler, Charalampos Aslanidis, and Gerd Schmitz[1]
Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, D-93053 Regensburg, Germany

Received April 19, 1997

A novel differentiation-dependent cDNA (DIF-2) has been isolated from human mononuclear phagocytes by differential display. The full-length cDNA was cloned and sequenced. DIF-2 consists of 156 amino acids and has a predicted isoelectric point of 8.84. The mRNA is expressed in freshly isolated monocytes and is downregulated significantly when monocytes are subjected to differentiation. A similar differentiation-dependent downregulation is observed in normal hepatocytes compared to undifferentiated HepG2 cells. The mRNA expression in monocytes is sensitive to lipopolysaccharide and ceramide which both strongly increase DIF-2 transcription, while lysophosphatidylcholine results in a weaker upregulation of DIF-2 expression. A DIF-2 homologous gene has been previously isolated from mouse fibroblasts and was shown to be a serum growth factor-inducible immediate early gene. Our results indicate that DIF-2 represents a gene which is regulated in differentiation processes and strongly responsive to lipopolysaccharide, ceramide and lysophosphatidylcholine.

Monocyte-derived macrophages play a major role in inflammatory disorders and in atherosclerosis. Actively phagocytosing macrophages are capable of enhanced uptake of cell debris and modified lipoproteins leading to foam cell formation (1,2). Inflammatory activated macrophages secrete a variety of cytokines and growth factors, thus influencing the mitotic and secretory activity of various cells (e.g. smooth muscle cells, T-lymphocytes) (2). Blood monocytes represent the circulating precursor cell pool for tissue macrophages and dendritic cells (DC) which are higly specialized in antigen presentation (3). Functional heterogeneity of cells at various stages of differentiation is closely linked to changes in specific gene expression. A number of proteins are upregulated during terminal maturation of monocytes to macrophages. The upregulation of a variety of scavenger receptors (e.g. class A, CD36), Fc- and complement receptors facilitates the elimination of modified lipoproteins and opsonized ligands from sites of inflammation and atherosclerotic lesions (4-6). Secretion of apolipoproteins (e.g. apoE) (7) and transfer proteins (e.g. CETP, PLTP, LBP) (8) enable an efficient removal and degradation of lipoprotein components and inflammatory agents from the cells. In addition, following differentiation, cytokines and proteinases are being secreted and influence the activity of various cells involved in inflammation, atherosclerosis and tissue remodelling. The decision whether macrophages function as antigen presenting cells in inflammation or as scavenger cells in the elimination of a variety of substrates is triggered by biological response modulators leading to transcriptional activation or repression of certain genes (9).

The effect of the proinflammatory stimulus lipopolysaccharide (LPS) on monocyte and macrophage activation is well characterized (10), while the responses to antiatherogenic (e.g. high density lipoprotein; HDL) and atherogenic lipoproteins (e.g. low density lipoprotein and its modified forms; LDL, oxLDL) and their lipid mediators, such as lysophosphatidylcholine (lysoPC), lysophosphatidic acid (lysoPA) (11,12) and ceramide (13) are subject of intensive investigations. LysoPC, a major phospholipid constituent of atherogenic lipoproteins, in particular of oxLDL, is generated from phosphatidylcholine through the action of phospholipase A_2 at sites of inflammation (11). The expression of a number of proteins involved in inflammatory and atherogenic processes is induced by modified LDL and its lipid components. In addition, lysoPC and

[1] Correspondence to: Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Franz-Josef-Strauß-Allee 11, D-93053 Regensburg, Germany. Fax: + 49 - 941 - 9446202. E-mail: Gerd.Schmitz@klinik.uni-regensburg.de.

0006-291X/97 $25.00

lysoPA potentiate the mitogenic activity of oxLDL for human macrophages (12).

Ceramide, another phospholipid derivative, is generated from lipoprotein or cell membrane sphingomyelin by the action of sphingomyelinase (SMase) and leads to an activation of ceramide-activated protein kinase (CAPK). It could be demonstrated, that the intracellular processes initiated by ceramide lead to cell differentiation and apoptosis (14). LPS can activate CAPK, without generating ceramide, suggesting that the LPS stimulation of cells mimics the second messenger function of ceramide (14). This is also supported by the obvious structural similarity between a portion of the lipid A region of LPS and ceramide (14) and by the similarity of ceramide- and LPS-induced signalling pathways (15). LPS stimulation of human mononuclear phagocytes is primarily mediated via the interaction of LPS and serum proteins, e.g. lipopolysaccharide binding protein (LBP) with the LPS-receptor (CD14), resulting in a cascade of intracellular reactions involving various kinases and the triggering of transcription factors (10,16).

The identification of genes that are either up- or downregulated during monocyte differentiation and upon stimulation with lysoPC, ceramide or LPS may contribute to the elucidation of molecular mechanisms involved in initial processes of inflammation and atherosclerosis and the accompanying functional changes. Here we report the identification of a novel cDNA from human monocytes by differential display that is downregulated during differentiation to macrophages and elaborate on the transcriptional regulation following stimulation with ceramide, lysoPC and LPS.

MATERIAL AND METHODS

Monocyte isolation and cultivation. Peripheral human mononuclear cells (MNC) from healthy volunteers were isolated by leukapheresis. Monocyte isolation from human MNC was performed by counterflow centrifugal elutriation as previously described (17). Freshly isolated monocytes were cultured on 15 cm^2 dishes (Falcon, Becton Dickinson, Heidelberg, FRG) at a density of 1×10^6 cells/ml in a specific macrophage medium (Macrophage-SFM-medium; Gibco, Eggenstein; FRG) supplemented with L-Glutamin without antibiotics. Following overnight incubation the monocytes were stimulated with LPS from *E.coli* 26B06 (500 ng/ml), C_2-ceramide (8 μM), L-α-lysophosphatidylcholine (100 μM) and phorbol-12-myristate-13-acetate (PMA) (160 nM) up to 24 hours. *In vitro* differentiation of monocytes to macrophages was performed as previously described (18). Macrophages were then incubated for 24 hours with acetylated LDL (acLDL) (100 μg/ml), HDL_3 (100 μg/ml), 10% lipoprotein-deficient serum (LPDS) and for 4 hours with LPS (500 ng/ml) in different experiments.

The human promonocytic cell lines THP-1, HL-60 and U937 were grown in culture in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Eggenstein, FRG). Cultured THP-1 cells were incubated with 160 nM PMA for up to 72 hours prior to RNA isolation (19). The preparation and chemical modification of the lipoproteins used in the experiments has been described elsewhere (20).

RNA isolation and Northern blot analysis. Total cellular RNA from monocytes, monocytic cell lines THP-1, U937, HL-60 and *in vitro* differentiated macrophages was isolated using the isothiocyanate/cesium chloride-ultracentrifugation method (21). For Northern blot analysis, 15 μg RNA/lane was separated on 1.2% agarose formaldehyde gels (22) and the nucleic acids blotted onto positively charged nylon membranes (GeneScreen Plus, DuPont, Bad Homburg, FRG). Hybridizations were performed using the non-radioactive DIG Labeling and Detection Kit from Boehringer (Mannheim, FRG).

Differential display (DD-RT/PCR). Differential display was performed essentially as previously described (23) with the following modifications. In brief, 0.2 μg total RNA, isolated from monocytes at various incubations, was reverse transcribed with specific anchored oligo-dT primers, using a commercially available kit (GeneAmp RNA PCR Core Kit, Perkin Elmer, Vaterstetten, FRG). The oligo-dT primers used had two additional nucleotides at their 3′ end consisting of an invariable A at the second last position (3′-end) and A, C, G or T at the last position to allow a subset of mRNAs to be reverse transcribed. Here, a 13-mer oligo-dT (T101: 5′-T_{11}AG-3′) was used in a 20 μl reaction at 2.5 μM concentration. One tenth of the cDNA was amplified in a 20-μl PCR reaction using the same oligo-dT primer and an arbitrary 10-mer upstream primer (D12: 5′-CTGCTTGATG-3′), 2.5 μM each, using 2.5 units of *Taq* DNA Polymerase and 1.25 mM $MgCl_2$. Amplification was for 40 cycles with denaturation at 94°C for 30 sec, annealing at 41°C for 1 min, and elongation at 72°C for 30 sec with a 5 min extension at 72°C following the last cycle. All PCR reactions were carried out in a Perkin Elmer 9600 thermocycler (Vaterstetten, FRG). PCR-products were separated on ready to use, 10% polyacrylamide gels with a 5% stacking gel (CleanGel Large-10/40, ETC, Kirchentellinsfurt, FRG) under non-denaturing conditions using the Multiphor II electrophoresis apparatus (Pharmacia, Freiburg, FRG). The DNA fragments were visualized by silverstaining of the gel as previously described (24,25).

Cloning and sequencing of differentially expressed cDNAs. cDNA bands of interest were cut out of the gel and DNA was isolated by boiling the gel slice for 10 min in 20 μl of water. A 4 μl aliquot was used for the following PCR-reaction in a 20-μl volume. The cDNA was reamplified using the same primer set and PCR conditions as above, except that the final dNTP concentration was 1 mM each. Reamplified cDNAs were cloned in the *Hinc*II-site of pUC18. Individual clones were sequenced on an automated fluorescence DNA sequencer using the AutoRead Sequencing Kit (Pharmacia, Freiburg, FRG) and were used as probes for Northern blot analysis.

5′-Rapid amplification of cDNA ends (RACE) - PCR. In order to identify the 5′-end of the cDNAs, 5′-RACE-Ready cDNA prepared from human leukocyte poly(A)-rich RNA (Clontech, Heidelberg, FRG) was amplified according to the recommendations of the manufacturer. A primary PCR reaction was performed with the provided anchor primer and the gene specific primer PAP1 (5′- CTTCCCACCGGGCCTAGCCCCAGCTGGG- 3′). A 1 μl aliquot of the initial PCR reaction served as template in a secondary PCR reaction (nested PCR) with the anchor primer and the nested gene specific primer PAP2 (5′- GCCTCGGTCTCTGTGCGCCTCGGTCCCG- 3′). PCR fragments were cloned in the *Hinc*II-site of pUC18 and sequenced.

RESULTS

Differentially expressed mRNAs during in vitro culturing of monocytes. In order to identify mRNAs that are induced during monocytic differentiation, differential display with total RNA isolated from freshly isolated monocytes and *in vitro* differentiated macrophages was performed. Preliminary analysis using four different oligo-dT primers and seven upstream primers (decamers) revealed that reproducible results were obtained when the oligo-dT primer T101 in combination

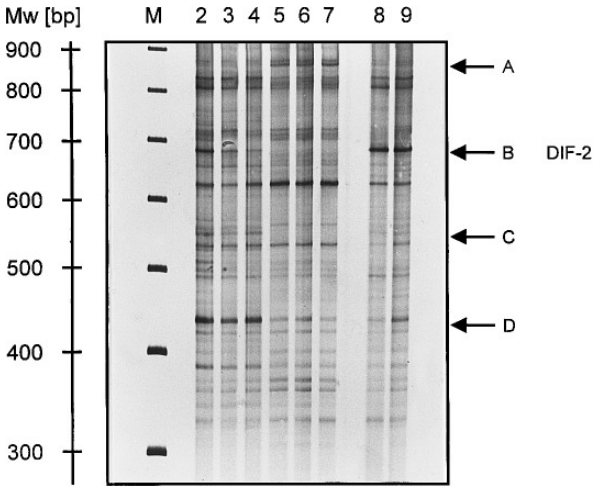

FIG. 1. Differential display of cDNAs from freshly isolated monocytes (lanes 2-4), *in vitro* differentiated macrophages (lanes 5-7) and monocytes stimulated with LPS - 500 ng/ml; 4 hours (lanes 8 and 9), separated on a silver-stained 10% polyacrylamide gel. DD-RT/PCR was performed on total RNA as described in Materials and Methods. Arrows indicate differentially expressed mRNAs in three independent RT/PCR-reactions. Lane M: molecular size standard.

with the decamer-primer D12 was used. Figure 1 shows the results from three independent PCR reactions from monocytic mRNA (lanes 2-4) compared to mRNA from *in vitro* differentiated macrophages (lanes 5-7) and in addition, mRNA from LPS-stimulated monocytes (4 hours, 500 ng/ml; lanes 8, 9). Three mRNAs are downregulated in macrophages (B=DIF-2, C and D) and one is upregulated (A). The most prominent difference was seen with DIF-2 which was absent in *in vitro* differentiated macrophages (lanes 5-7) and strongly upregulated in LPS-stimulated monocytes compared to unstimulated monocytes (lanes 8, 9). This band was cut out of the gel, reamplified, cloned and sequenced. Northern blot analysis with the cloned fragment as a probe revealed differentiation-dependent expression of a 1.2 kb mRNA (Fig. 2 A). As can be seen in figure 2 A, monocytes (lane 1) gave a stronger signal than *in vitro* differentiated macrophages (lane 3). LPS had a strong stimulatory effect on the expression of DIF-2 in monocytes (lane 2) as well as in macrophages (lane 4). Northern blot analysis of RNA from freshly isolated monocytes and *in vitro* differentiated macrophages incubated with LPDS, acLDL or HDL_3 (Fig. 2 B) revealed a strong hybridization signal with the DIF-2 probe in monocytes (lane 1) compared to differentiated cells (lane 2). RNA of cholesterol-depleted macrophages (LPDS, lane 3) showed a moderate increased hybridization signal compared to cells incubated with acLDL (lane 4) or HDL_3 (lane 5) (Fig. 2 B).

DNA- and protein sequence of DIF-2. Cloning and sequencing of the cDNA fragment obtained from DD-RT/PCR (Fig. 1) revealed a 609 bp genuine sequence. This sequence could be extended to full-length cDNA (1230 bp) by 5′-RACE-PCR. The complete 1230 bp sequence was in agreement with the size observed on Northern blots (Fig. 2 A). As shown in figure 3 an open reading frame of 468 bp codes for a protein of 156 amino acids with a predicted isoelectric point of 8.84. The presumed start codon ATG is embedded in a sequence homologous to the Kozak consensus sequence (26). The DIF-2 clone contains five conserved AT-rich sequences. A putative polyadenylation signal (AATAAA) was present at position +1196 to +1201. Sites for potential phosphorylation and N-glycosylation are also present within the protein (Fig. 3).

Comparison of the full-length DIF-2 cDNA sequence with DNA sequences from GeneBank and EMBL database demonstrated high homology to mouse gly96 sequence and identified partial human sequences stored as expressed sequence tags. The mouse gly96 was identified as a growth factor-inducible immediate early gene expressed in fibroblasts encoding a short-lived, glycosylated protein of 153 amino acids (27). Human and mouse gly96 have an overall identity of 69% at the nucleotide sequence and 73% at the protein level.

Effects of ceramide, LPS, LysoPC and PMA on DIF-2 expression. In order to investigate the effects of various biological response modifiers on monocyte DIF-2 expression we have analyzed its time-dependent expression by Northern blots in the presence or absence of these modifiers (Fig. 4 A). Freshly isolated monocytes that were kept in a specific macrophage medium overnight gave strong hybridization with a DIF-2 probe

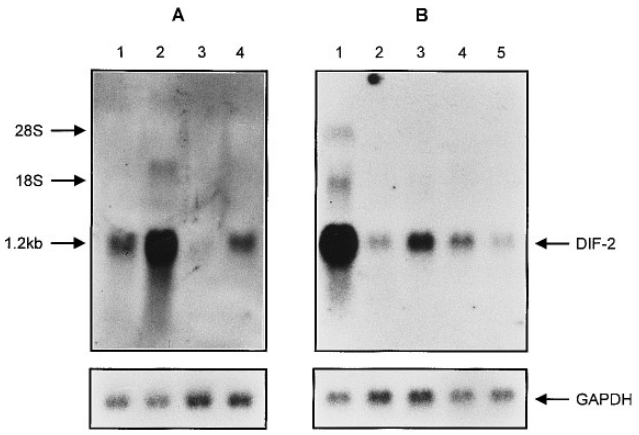

FIG. 2. Northern blot analysis of DIF-2. A total of 15 μg RNA was electrophoresed on a formaldehyde agarose gel. Labeled DIF-2 cDNA-fragment (obtained from the DD-RT/PCR) was used as a probe. (A) Lane 1: monocytes, lane 2: monocytes stimulated with LPS (500 ng/ml, 4 hours), lane 3: macrophages, lane 4: macrophages stimulated with LPS (500 ng/ml, 4 hours). (B) Lane 1: monocytes, lane 2: *in vitro* differentiated macrophages, lane 3: macrophages incubated with 10% LPDS (24 hours), lane 4: macrophages incubated with acLDL (100 μg/ml, 24 hours), lane 5: macrophages incubated with HDL_3 (100 μg/ml, 24 hours). Even RNA loading in the individual lanes was assessed by hybridizations with a GAPDH cDNA probe.

```
CTCGGCTCACCATGTGTCACTCTCGCAGCTGCCACCCGACCATGACCATCCTGCAGGCCC
          M  C  H  S  R  S  C  H  P  T  M  T  I  L  Q  A  P

CGACCCCGGCCCCCTCCACCATCCCGGGACCCCGGCGGGGCTCCGGTCCTGAGATCTTCA
T  P  A  P  S  T  I  P  G  P  R  R  G  S  G  P  E  I  F  T

CCTTCGACCCTCTCCCGGAGCCCGCAGCGGCCCCTGCCGGGCGCCCCAGCGGCTCTCGCG
F  D  P  L  P  E  P  A  A  A  P  A  G  R  P  S  G  S  R  G

GGCACCGAAAGCGCAGCCGCAGGGTTCTCTACCCTCGAGTGGTCCGGCGCCAGCTGCCAG
H  R  K  R  S  R  R  V  L  Y  P  R  V  V  R  R  Q  L  P  V

TCGAGGAACCGAACCCAGCCAAAAGGCTTCTCTTTCTGCTGCTCACCATCGTCTTCTGCC
E  E  P  N  P  A  K  R  L  L  F  L  L  L  T  I  V  F  C  Q

AGATCCTGATGGCTGAAGAGGGTGTGCCGGCGCCCCTGCCTCCAGAGGACGCCCCTAACG
I  L  M  A  E  E  G  V  R  A  P  L  P  P  E  D  A  P  N  A

CCGCATCCCTGGCGCCCACCCCTGTGTCCCCCGTCCTCGAGCCCTTTAATCTGACTTCGG
A  S  L  A  P  T  P  V  S  P  V  L  E  P  F  N  L  T  S  E

AGCCCTCGGACTACGCTCTGGACCTCAGCACTTTCCTCCAGCAACACCCGGCCGCCTTCT
P  S  D  Y  A  L  D  L  S  T  F  L  Q  Q  H  P  A  A  F  *

AACTGTGACTCCCCGCACTCCCCAAAAAGAATCCGAAAAACCACAAAGAAACACCAGGCG
TACCTGGTGCGCGAGAGCGTATCCCCAACTGGGACTTCCGAGGCAACTTGAACTCAGAAC
ACTACAGCGGAGACGCCACCCGGTGCTTGAGGCGGGACCGAGGCGCACAGAGACCGAGGC
GCATAGAGACCGAGGCACAGCCCAGCTGGGGCTAGGCCCGGTGGGAAGGAGAGCGTCGTT
AATTTATTTCTTATTGCTCCTAATTAATATTTATATGTATTTATGTACGTCCTCCTAGGT
GATGGAGATGTGTACGTAATATTTATTTTAACTTATGCAAGGGTGTGAGATGTTCCCCCT
GCTGTAAATGCAGGTCTCTTGGTATTTATTGAGCTTTGTGGGACTGGTGGAAGCAGGACA
CCTGGAACTGCGGCAAAGTAGGAGAAGAAATGGGGAGGACTCGGGTGGGGGAGGACGTCC
CGGCTGGGATGAAGTCTGGTGGTGGGTCGTAAGTTTAGGAGGTGACTGCATCCTCCAGCA
TCTCAACTCCGTCTGTCTACTGTGTGAGACTTCGGCGGACCATTAGGAATGAGATCCGTG
AGATCCTTCCATCTTCTTGAAGTCGCCTTTAGGGTGGCTACGAGGTAGAGGGGTGGGGGT
TGGTGGGCTGTCACGGAGCGACTGTCGAGATCGCCTAGTATGTTCTGTGAACACAAATAA
AATTGATTTACTGTCTGCTAAAAAAAAAAA
```

FIG. 3. The cDNA and deduced amino acid sequences of DIF-2 are shown. DNA sequence determined by 5′-RACE-PCR is underlined. Putative phosphorylation and N-glycosylation sites appear in italics and in bold, and RNA stabilization domains in the 3′ untranslated region (ATTTA) are double underlined. A putative polyadenylation signal (AAATAA) is emphasized in bold. *, termination codon.

(lane 1) compared to cells that were kept in the same medium for additional 24 hours (lane 2).

The presence of various stimuli (C_2-ceramide, LPS, lysoPC and PMA) lead to an upregulation of DIF-2 expression in monocytes. LPS (lanes 3-5) and ceramide (lanes 6-8) had the same effects on the mRNA expression of DIF-2. Both stimuli increased the mRNA abundance strongly during the whole incubation time, with maximal peak levels after 4 hours (LPS, lane 4; ceramide, lane 7). LPS (500 ng/ml) leads to stronger hybridization signals compared to ceramide (8 μM). Stimulation of monocytes with lysoPC (lanes 9-11) resulted in only transient upregulation of the gene expression and declined after 24 hours of incubation (lane 11) in comparison to LPS and C_2-ceramide. PMA (lanes 12-14), a protein kinase C activator, leads to an upregulation of the mRNA with maximal effects at 24 hours (lane 14).

Tissue and cell specific expression of DIF-2. DIF-2 mRNA is most abundant in freshly isolated monocytes, lymphocytes and keratinocytes (HaCaT) and has an expression at a low level in kidney, intestine, fibroblasts and *in vitro* differentiated macrophages. No detectable mRNA was observed in lung and liver (Fig. 4 B). A strong hybridization signal was detected in RNA isolated from the HepG2 liver tumor cell line compared to fully differentiated human hepatocytes (Fig. 4 B, lanes 3 and 5).

Analysis of the promonocytic cell line THP-1 for DIF-2-mRNA expression upon incubation with PMA (3, 6, 24, 48 and 72 hours) resulted in transcriptional activation with a maximum of mRNA expression at 24 hours. This upregulation at 24 hours is followed by a downregulation of DIF-2 mRNA at 48 and 72 hours. Unstimulated U937 and HL-60 cells did not result in detectable hybridization signals for DIF-2 (data not shown).

DISCUSSION

The differentiation of monocytes to macrophages and their activation play a key role in immunological responses, inflammation and the progression of atherosclerosis. Therefore it is not surprising that macrophages display a range of functional and morphological phenotypes in various tissues and body cavities (2). Using the differential display technique we have identified a novel gene (DIF-2) which is strongly expressed in freshly isolated monocytes and significantly downregulated during the differentiation to macrophages.

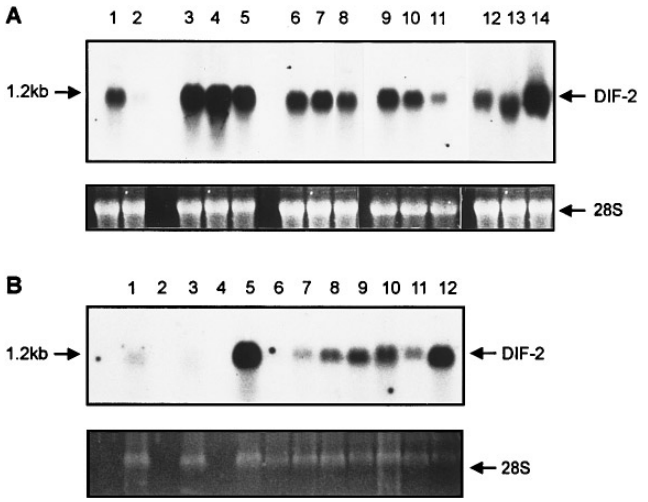

FIG. 4. (A) Effects of LPS, C_2-ceramide, lysoPC and PMA on DIF-2 expression. Following overnight incubation (time: 0 hours) freshly isolated monocytes were stimulated with 500 ng/ml LPS, 8 μM C_2-ceramide, 100 μM lysoPC and 160 nM PMA for various times (1, 4, 24 hours) and total RNA was isolated and subjected to Northern blot analysis with DIF-2 cDNA-fragment. 1: control 0 hours; 2: control 24 hours; 3: LPS 1 hour; 4: LPS 4 hours; 5: LPS 24 hours; 6: ceramide 1 hour; 7: ceramide 4 hours; 8: ceramide 24 hours; 9: lysoPC 1 hour; 10: lysoPC 4 hours; 11: lysoPC 24 hours; 12: PMA 1 hour; 13: PMA 4 hours; 14: PMA 24 hours. (B) Tissue distribution of DIF-2 mRNA. Tissues analyzed were kidney (1), liver (3), HepG2 cells (5), lung (6), intestine (7), fibroblasts (8), keratinocytes (HaCaT) (9), monocytes (10), macrophages (11), lymphocytes (12). Lanes 2 and 4 contain no RNA. The DIF-2 mRNA is indicated by an arrow. Comparable loading of the agarose gel is demonstrated by the equal intensities of the ethidium bromide-stained 28S rRNA.

A 1230 bp full-length cDNA coding for a novel 156 amino acid long protein was isolated. DIF-2 is highly homologous to a mouse gene (gly96), which was originally identified as an immediate early gene (27). Gly96 has been shown to be expressed during the G_0/G_1 transition of the cell cycle in quiescent Balb/c 3T3 fibroblasts, and it was also suggested that its expression is regulated through a PKC-independent pathway (27). Charles et al. (27) demonstrated high expression of the murine mRNA in mouse uterus, lung and testis, while we detected high mRNA levels of the human mRNA in human monocytes, lymphocytes, keratinocytes (HaCaT) and HepG2 cells, but not in lung. DIF-2 is highly expressed in freshly isolated monocytes and is downregulated in *in vitro* differentiated macrophages. An involvement of DIF-2 in differentiation processes is further supported by the observed high expression in the hepatocyte-derived tumor cell line HepG2 in comparison to fully differentiated normal hepatocytes.

Oxidized lipoproteins and their atherogenic phospholipid component lysoPC have been shown to play a role in chemotaxis, cell growth and differentiation (14,15). Macrophages resemble terminally differentiated cells of the mononuclear phagocyte lineage and do not further proliferate without growth stimulation. OxLDL can stimulate the growth of human macrophages, and lysoPC may play an essential role in the mitogenic activity (12). We have analyzed the effect of lysoPC on the expression of DIF-2 in monocytes. Compared to non-lysoPC exposed monocytes, which show strong downregulation of DIF-2 during differentiation, lysoPC-incubated cells revealed a delayed and less prominent downregulation of DIF-2, likely due to the mitogenic activity of the stimulus. LysoPC is capable to potentiate the mitogenic activity of oxLDL for human macrophages (12), implicating that the more moderate downregulation of DIF-2 may lead to a persistence of the monocytic state.

The high expression of DIF-2 in monocytes and downregulation during differentiation implicates that DIF-2 may be of relevance for monocyte specific functions. This is supported by the observation that LPS, a potent stimulus for monocytes and macrophages, has a significant effect on the mRNA expression in monocytes but not in macrophages. Wright et al. have suggested, that LPS may stimulate cells by mimicking the second messenger function of ceramide (28). LPS and ceramide lead to comparable DIF-2 expression levels and a maximal upregulation after 4 hours in stimulated monocytes. These observations are in agreement with the postulated functional similarity of ceramide and the lipid A component of LPS and the suggested common mode of action (14,15).

Treatment of cells with PMA results in a rapid induction of immediate early gene expression (29). After PMA incubation of freshly isolated monocytes a steady increase in the DIF-2 mRNA resulted in maximal expression at 24 hours. This seems to contradict the assumption of being an immediate early gene, as proposed by Charles et al. (27). In the monocytic cell line THP-1, PMA treatment resulted in a loss of proliferation and initiated differentiation (30). Unstimulated THP-1, U937 and HL-60 cell lines do not express detectable amounts of DIF-2. PMA activation of THP-1 cells induces cell differentiation to more mature cells and leads to an upregulation of DIF-2 mRNA with a peak maximum at 24 hours. This can be explained by the fact, that THP-1 cells represent precursors of monocytes that increase DIF-2 expression during differentiation.

Whether DIF-2 is involved in basic differentiation processes, as implicated by the observations in monocytic and hepatic cells, needs further investigation. In addition, analyses are underway to elucidate whether DIF-2 expression in native macrophages from lesions differs from that observed in *in vitro* differentiated macrophages. Cloning and overexpression of the protein and the generation of antibodies will contribute to the functional characterization and the relevance of altered mRNA expression seen in various tissues.

ACKNOWLEDGMENTS

This study was supported in part by the *Deutsche Forschungsgemeinschaft* (AN 111/6-1, project 1) and *Bayer AG*, Leverkusen (Germany). The expert technical assistance of S. Potra is appreciated.

REFERENCES

1. Ross, R. (1993) *Nature* **362,** 801–806.
2. Rutherford, M. S., Witsell, A., and Schook, L. B. (1993) *J. Leukoc. Biol.* **53,** 602–618.
3. Steinman, R. M. (1991) *Annu. Rev. Immunol.* **9,** 271–296.
4. Krieger, M., and Herz, J. (1994) *Annu. Rev. Biochem.* **63,** 601–637.
5. Huh, H. Y., Pearce, S. F., Yesner, L. M., Schindler, J. L., and Silverstein, R. L. (1996) *Blood* **87,** 2020–2028.
6. Stanton, L. W., Tyler, White, R., Bryant, C. M., Protte, A. A., and Endemann, G. (1993) *J. Biol. Chem.* **268,** 11811–11816.
7. Basheeruddin, K., Rechtoris, C., and Mazzone, T. (1992) *J. Biol. Chem.* **267,** 1219–1224.
8. Jiang, X. C., Bruce, C., Cocke, T., Wang, S., Boguski, M., and Tall, A. R. (1995) *Biochem.* **34,** 7258–7263.
9. Ohlsson, B. G., Englund, M. C., Karlsson, A. L., Knutsen, E., Erixon, B., Skribeck, H., Liu, Y., Bondjers, G., and Wiklund, O. (1996) *J. Clin. Invest.* **98,** 78–89.
10. Sweet, M. J., and Hume, D. A. (1996) *J. Leukoc. Biol.* **60,** 8–26.
11. Kume, N., Cybulsky, M. I., and Gimbrone, M. A. Jr. (1992) *J. Clin. Invest.* **90,** 1138–1144.
12. Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matsumura, T., Kobori, S, Shichiri, M., and Horiuchi, S. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16,** 600–605.
13. Ballou, L. R., Laulederkind, S. J. F., Rosloniec, E. F., and Raghow, R. (1996) *Biochim. Biophys. Acta* **1301,** 273–287.
14. Joseph, C. K., Wright, S. D., Bornmann, W. G., Randolph, J. T., Kumar, E. R., Bittman, R., Liu, J., and Kolesnick, R. N. (1994) *J. Biol. Chem.* **269,** 17606–17610.

15. Barber, S. A., Detore, G., McNally, R., and Vogel, S. N. (1996) *Infect. Immun.* **64,** 3397–3400.
16. Hailman, E., Vasselon, T., Kelley, M., Busse, L. A., Hu, M. C. T., Lichenstein, H. S., Detmers, P. A., and Wright, S. D. (1996) *J. Immunol.* **156,** 4384–4390.
17. Fogelman, A. M., Seager, J., Hokom, M., and Edwards, P. A. (1979) *J. Lipid Res.* **20,** 379–388.
18. Müller, G., Kerkhoff, C., Hankowitz, J., Pataki, M., Kovacs, E., Lackner, K. J., and Schmitz, G. (1993) *Arterioscler. Thromb.* **13,** 1317–1326.
19. Lee, B. S., Underhill, D. M., Crane, M. K., and Gluck, S. L. (1995) *J. Biol. Chem.* **270,** 7320–7329.
20. Schmitz, G., Fischer, H., Beuck, M., Hoecker, K. P., and Robenek, H. (1990) *Arterioscler.* **10,** 1010–1019.
21. Chirgwin, J. J., Przbyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *In* Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Liang, P., and Pardee, A. B. (1992) *Science* **257,** 967–971.
24. Bassam, B. J., Caetano-Anolles, G., and Gresshoff, P. M. (1991) *Anal. Biochem.* **196,** 80–83.
25. Lohmann, J., Schickle, H. P., and Bosch, T. C. G. (1995) *BioTechniques* **18,** 200–202.
26. Kozak, M. (1987) *Nucl. Acids Res.* **15,** 8125–8148.
27. Charles, C. H., Yoon, J. K., Simske, J. S., and Lau, L. F. (1993) *Oncogene* **8,** 796–801.
28. Wright, S. D., and Kolesnick, R. N. (1995) *Immunol. Today* **16,** 297–301.
29. Dunnmon, P. M., Iwaki, K., Henderson, S. A., Sen, A., and Chien, K. R. (1990) *J. Mol. Cell. Cardiol.* **22,** 901–910.
30. Tsuchiya, S., Kabayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. (1982) *Cancer Res.* **42,** 1530–1536.