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cDNA-CLONING, SEQUENCING AND EXPRESSION IN GLUCOCORTICOID-STIMULATED QUIESCENT SWISS 3T3 FIBROBLASTS OF MOUSE LIPOCORTIN I

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We isolated and sequenced mouse lipocortin I cDNA clones from a lambda gt10 cDNA library prepared from Swiss 3T3 mRNA. The homology with human lipocortin I at the amino acid level is 86%. When confluent layers of Swiss 3T3 cells were stimulated with 10% fetal calf serum, expression of lipocortin I was strongly stimulated. In parallel, DNA synthesis was induced with a peak at 24 hours after glucocorticoid treatment indicating induction of cell proliferation. In the absence of serum glucocorticoid treatment provoked neither induction of DNA synthesis nor expression of lipocortin I. We conclude that serum contains an unidentified factor, which acts synergistically with glucocorticoids on cell proliferation and lipocortin I expression.

Lipocortins are a family of phospholipase A_2 (PLA_2) inhibitory proteins, comprising at least six different members (1,11). Their synthesis was found to be induced by glucocorticoids in a variety of tissues and cells (2,3) thereby increasing the inhibition of PLA_2. The PLA_2 inhibitory activity of lipocortins was reported to be regulated via phosphorylation by various kinases including cyclic AMP dependent protein kinases, protein kinase C and tyrosine protein kinases (4,5,6). Phosphorylated lipocortin has been shown to be inactive in inhibiting PLA_2 activity (4).

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ABBREVIATIONS: PLA_2, phospholipase A_2; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PBS, Dulbecco's phosphate buffered saline (0.9% NaCl); EDTA, ethylendiamine tetraacetate, sodium salt; FA, fluocinolone acetonide; SSC, standard saline citrate; DMEM, Dulbecco's modified Eagle's medium; ETYA, 5,8,11,14-eicosatetraynic acid; BSA, bovine serum albumin.

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The biochemical mechanism of PLA_2 inhibition is still unclear. Using a cell-free system it has been shown (14) that the inhibitory effect of lipocortin is due to sequestering of the phospholipid substrate leading to "substrate depletion" (13) rather than to direct interaction with PLA_2 (4). It is also unknown, whether lipocortins act inside or outside of the cell. Inhibition of PLA_2 by glucocorticoid prevents the liberation of arachidonic acid from cellular phospholipids and, as a consequence, the formation of prostaglandins along the cyclooxygenase and of leukotriens along the lipoxygenase pathway. These arachidonic acid metabolites have been shown to be involved in a variety of different cellular responses, in particular in the early events of growth factor-induced cell proliferation (22). The results obtained with Swiss 3T3 fibroblast cultures are conflicting (20,22). Thus, glucocorticoid treatment has been reported to lead to reduced arachidonic acid release and decreased cell proliferation (20,22). On the other hand, glucocorticoids have been shown to stimulate DNA synthesis and mitotic activity in Swiss 3T3.

Here we report on the induction of both lipocortin I gene expression and cell proliferation in Swiss 3T3 by the glucocorticoid analogue fluocinolone acetonide using a cloned and sequenced mouse lipocortin I cDNA probe. Our data demonstrate that cell proliferation in 3T3 cultures is accompanied by increased rather than by decreased lipocortin I expression and is insensitive to inhibitors of arachidonic acid metabolism.

MATERIALS AND METHODS

cDNA library, screening: An oligo (dT)-primed lambda gt10 cDNA library was prepared from poly(A) + RNA from Swiss 3T3 fibroblasts, grown in the presence of 10% fetal calf serum (FCS). 10^7 independent clones were obtained. The first screening was performed using two synthetic oligonucleotides designed after the published human lipocortin I sequence (8), stretching from position 247 to 286 and from position 337 to 370. The screening was performed under the following conditions: 6 x SSC; 50 mM KH_2PO_4/Na_2HPO_4, pH 6.8.; 5 x Denhardt hybridization buffer at 37°C (12). The washing was performed in 3 M tetramethylammoniumchloride; 50 mM Tris-HCl, pH 8.0; 2 mM EDTA; 0.1% SDS at 48°C.
Sequencing: Nucleotide sequence was determined using the didesoxy sequencing method (18).
DNA subcloning: Insert DNA from phages was subcloned into plasmid pUC8 (Pharmacia, Freiburg, FRG) using standard techniques (12).

Northern blot analysis: For northern blot analysis cells were washed three times with PBS and lysed in a NP 40-containing buffer. The nuclei were pelleted at 1500 g. Subsequently, the cytoplasm was extracted with phenol, phenol/chloroform (1:1) and chloroform. RNA was precipitated with ethanol, washed, dried and resuspended in H_2O at 1 µg/µl. 5 µg RNA per slot were electrophoretically separated on 1% agarose gels containing 2.2 M formaldehyde. The gel was stained with ethidium bromide, destained, photographed, transferred to Gene Screen Plus membranes (NEN) and hybridized under stringent conditions (12) with a nicktranslated cDNA insert of mouse lipocortin I.
Cell culture: For stock cultures Swiss 3T3 cells (Flow Laboratories, Meckenheim, FRG) were grown in 100 mm dishes (Falcon) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Conco, Wiesbaden, FRG). For treatment 1.5×10^6 cells were grown in 24.5 x 24.5 cm dishes (Nunc, Roskilde, Denmark) for 6 days to reach confluence. Fresh medium supplemented with 0.1 µM fluocinolone acetonide (Sigma, St. Louis, USA) in 10% FCS or acetone in 10% FCS was added at zero time. Cells were harvested at the times indicated.
Determination of DNA synthesis: The rate of DNA synthesis was determined by pulse-labelling the cells with (^{3}H)-thymidine (5 µCi/ml; DuPont, Boston, USA) for one hour. DNA was isolated according to the Schmidt-Thannhauser procedure (9) and assayed by the Burton method (10) with calf thymus DNA as a standard.

RESULTS

A 3T3 cDNA library was constructed in the vector lambda gt10. Upon screening with two ^{32}P-labelled synthetic oligonucleotide probes designed after the published human lipocortin I sequence, one positive clone was isolated from this library and sequenced. As shown in Fig. 1, there is one large open reading frame region of 1023 nucleotides coding for the 341 carboxyterminal amino acids of mouse lipocortin I. The incomplete untranslated 3'-region consists of 307 nucleotides without poly(A) tail. The nucleotide sequence of the coding region was aligned with the appropriate region of published human and rat lipocortin I. We found a 86% homology to human (8) and 93% to rat (25,26) lipocortin I. The deduced amino acid sequence showed 86% homology to the human and 92% to the rat sequence.
Using the mouse lipocortin I cDNA we were able to study lipocortin expression in Swiss 3T3 fibroblasts by Northern blot analysis. Addition of fluocinolone acetonide (FA), the most active synthetic glucocorticoid, to confluent cultures grown in DMEM 10% FCS resulted in an increase of lipocortin I mRNA expression with a peak at 36 hours after treatment (Fig. 2a,b). Parallel to lipocortin mRNA expression we found an increase in DNA synthesis with a peak at 24 hours (Fig. 3). The same experiment carried out

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   1 GAATTCCTCAAGCAGGCCCGTTTTCTTGAAAATCAAGAACAGGAATATGTTCAAGCTGTA
     GluPheLeuLysGlnAlaArgPheLeuGluAsnGlnGluGlnGluTyrValGlnAlaVal

  61 AAATCATACAAAGGTGGTCCTGGGTCAGCAGTGAGCCCCTACCCTTCCTTCAATGTATCC
     LysSerTyrLysGlyGlyProGlySerAlaValSerProTyrProSerPheAsnValSer

 121 TCGGATGTTGCTGCCTTGCACAAAGCTATCATGGTTAAAGGTGTGGATGAAGCAACCATC
     SerAspValAlaAlaLeuHisLysAlaIleMetValLysGlyValAspGluAlaThrIle

 181 ATTGACATTCTTACCAAGAGGACCAATGCTCAGCGCCCAAGGATCAAGGCCGCGTACTTA
     IleAspIleLeuThrLysArgThrAsnAlaGlnArgProArgIleLysAlaAlaTyrLeu

 241 CAGGAGAATGGAAAGCCCTTGGATGAAGTCTTGAGAAAAGCCCTTACAGGCCACCTGGAG
     GlnGluAsnGlyLysProLeuAspGluValLeuArgLysAlaLeuThrGlyHisLeuGlu

 301 GAGGTTGTTTTGGCTATGCTAAAAACTCCAGCTCAGTTTGATGCAGATGAACTCCGTGGT
     GluValValLeuAlaMetLeuLysThrProAlaGlnPheAspAlaAspGluLeuArgGly

 361 GCCATGAAGGGACTTGGAACAGATGAAGACACTCTCATTGAGATTTTGACAACAAGATCT
     AlaMetLysGlyLeuGlyThrAspGluAspThrLeuIleGluIleLeuThrThrArgSer

 421 AACGAACAGATCAGAGAGATTAATAGAGTCTACAGAGAAGAGCTGAAAAGAGATCTGGCC
     AsnGluGlnIleArgGluIleAsnArgValTyrArgGluGluLeuLysArgAspLeuAla

 481 AAAGACATCACTTCAGATACATCTGGAGACTTTCGGAAAGCCTTGCTTGCTCTTGCCAAG
     LysAspIleThrSerAspThrSerGlyAspPheArgLysAlaLeuLeuAlaLeuAlaLys

 541 GGTGACCGTTGTCAGGACTTGAGTGTGAATCAAGATTTGGCTGATACAGATGCCAGGGCT
     GlyAspArgCysGlnAspLeuSerValAsnGlnAspLeuAlaAspThrAspAlaArgAla

 601 TTGTATGAAGCTGGAGAAAGGAGAAAGGGGACAGACGTGAACGTGTTCCATACAATTCTG
     LeuTyrGluAlaGlyGluArgArgLysGlyThrAspValAsnValPheHisThrIleLeu

 661 ACCAGTAGGAGCTTTCCTCATCTTCGCAGAGTGTTTCAGAATTACGGAAAGTACAGTCAA
     ThrSerArgSerPheProHisLeuArgArgValPheGlnAsnTyrGlyLysTyrSerGln

 721 CATGACATGAACAAAGCTCTGGATCTGGAACTGAAGGGTGACATTGAGAAGTGCCTCACA
     HisAspMetAsnLysAlaLeuAspLeuGluLeuLysGlyAspIleGluLysCysLeuThr

 781 ACCATCGTGAAGTGTGCCACCAGCCATCCAGCTTTCTTTGCCGAGAAGCTGTACGAAGCC
     ThrIleValLysCysAlaThrSerHisProAlaPhePheAlaGluLysLeuTyrGluAla

 841 ATGAAGGGTGCCGGAACTCGCCATAAGGCATTGATCAGGATTATGGTCTCCCGTTCGGAA
     MetLysGlyAlaGlyThrArgHisLysAlaLeuIleArgIleMetValSerArgSerGlu

 901 ATTGACATGAATGAAATCAAAGTATTTTACCAGAAGAAGTATGGAATCTCTCTTTGCCAA
     IleAspMetAsnGluIleLysValPheTyrGlnLysLysTyrGlyIleSerLeuCysGln

 961 GCCATCCTGGATGAAACCAAAGGAGACTATGAAAAAATCCTGGTGGCTCTGTGTGGTGGA
     AlaIleLeuAspGluThrLysGlyAspTyrGluLysIleLeuValAlaLeuCysGlyGly

1021 AACTAGACATCCCAACTACTCTGCAAGATTCTGAGGAGAATGTCTCTTAGCTGTTGTTTT
     ASN***

1081 CCTTCTCTTGCATGGGCTTAAGTAGGAAAGTTGCTTTGGCAGATAAGTCTAATTACCTGC

1141 TTTGAATAATATAGCCTATAAATAGATTTTACATCATTACTCTGTACAATAGAGAAATAC

1201 TTGTTTTGTTAATTATGTTTATCCCAAATTATAAAGCCCCATAAGCAAGTCACTTTGGTA

1261 CCATTCCTGAGAAAGAAGTTTACATATAATAAAATAAAACAATTTTATAGACAAAAAAAC

1321 GAATTCCCGG
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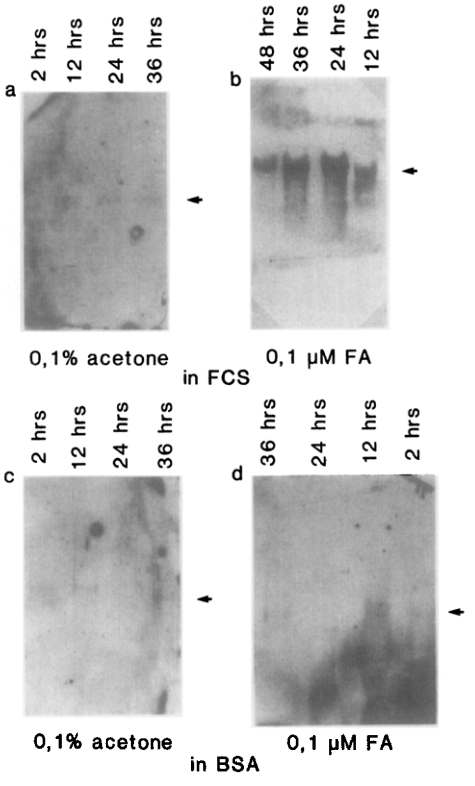
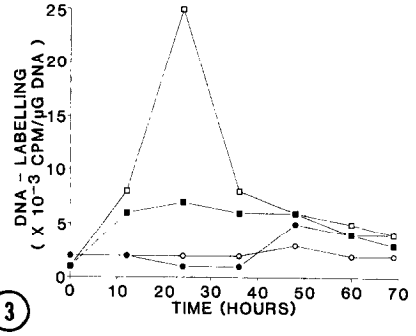
Fig. 1: cDNA nucleotide sequence and deduced amino acid sequence of mouse lipocortin I.
The nucleotide sequence represents a part of the cloned cDNA sequence.

with cells grown in DMEM containing BSA (1 mg/ml) instead of serum did not result in an induction of lipocortin mRNA expression or cell proliferation (Fig. 2c/d).

To decide whether prostaglandin synthesis is a prerequisite for glucocorticoid-induced lipocortin I expression and cell proliferation we treated 3T3 cells with either indomethacin (10 µM), an inhibitor of prostaglandin synthethase or ETYA (10 µM), an inhibitor of lipoxygenase of prostaglandin



(2)



(3)

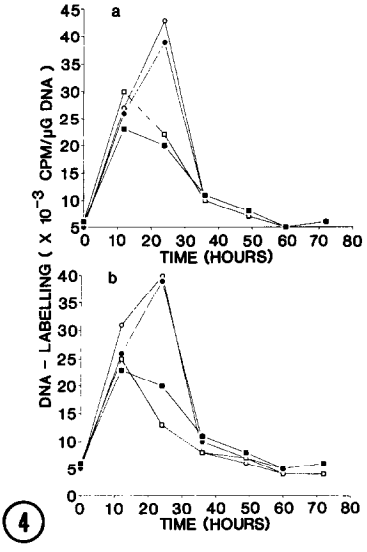
Fig. 2: Effect of FA and serum on lipocortin I mRNA-expression in Swiss 3T3 fibroblasts.
0.1 µM FA (b,d) or 0.1% acetone (controls, a,c) was added together with DMEM/10% FCS (a,b) or DMEM/BSA, 1 mg/ml (c,d) to confluent cultures at zero time. RNA was isolated and analyzed by Northern blot hybridization at the times indicated. A 1.3 kb fragment of mouse lipocortin I cDNA (Fig. 1) subcloned into pUC8 was used as a nick-translated probe. Arrow: position of lipocortin I-mRNA.

Fig. 3: Effect of FA and serum on DNA synthesis of Swiss 3T3 fibroblasts.
0.1 µM FA/0.1% acetone (open symbols) or acetone (0.1%, closed symbols) was added together with DMEM/10% FCS (□, ■) or DMEM/BSA, 1 mg/ml (o,●) to confluent 3T3 cultures at zero time. Pulse-labelling with (^{3}H)-thymidine was carried out at the times indicated. Each point represents the mean of 3 values (deviation $\leq$12%).

synthethase, together with FA (0.1 µM) in DMEM containing 10% serum. Indomethacin or ETYA neither blocked glucocorticoid-induced cell proliferation (Fig. 4) and lipocortin expression (Fig. 5) nor serum-induced cell proliferation (Fig. 4).

DISCUSSION

Using two synthetic oligonucleotides designed after the published human lipocortin I-sequence, we have cloned mouse lipocortin I complementary DNA.



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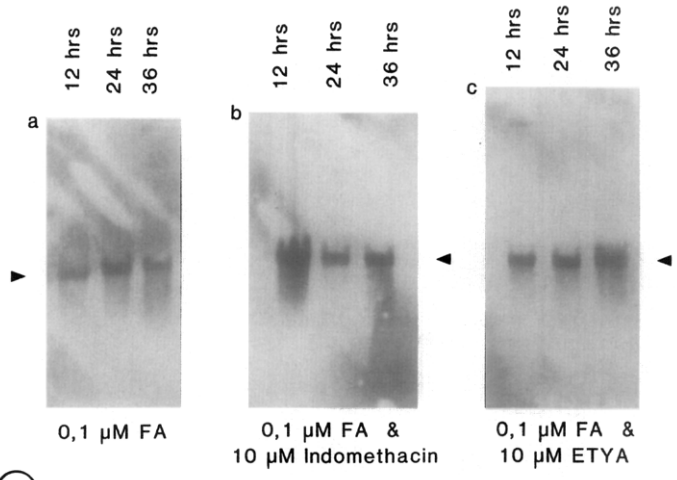

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Fig. 4: Effect of indomethacin (a) or ETYA (b) on serum- or FA-induced DNA-synthesis in Swiss 3T3 fibroblasts.
Together with DMEM/10% FCS the following additions were made at zero time:
0.1% acetone (■), 0.1 µM FA/0.1% acetone (●), 10 µM indomethacin (□), 10 µM ETYA (□), FA + indomethacin (o), FA + ETYA (o).
Pulse-labelling with (^{3}H)-thymidine was carried out at the times indicated. Each point represents the mean of 3 values (deviation ≤16%).

Fig. 5: Effect of indomethacin or ETYA on lipocortin I mRNA-expression in serum- and FA-stimulated Swiss 3T3 fibroblasts.
Together with DMEM/10% FCS the following additions were made at zero time:
0.1% FA/0.1% acetone (a), 0.1 µM FA+ 10 µM indomethacin (b), 0.1 µM FA + 10 µM ETYA (c). Northern blot hybridization was carried out as described for Fig. 3 at the times indicated. Arrow: position of lipocortin I mRNA.

Both the nucleotide and the deduced amino acid sequences turned out to be strongly homologous to but not identical with those of rat and human lipocortin I (8,25,26). The glucocorticoid fluocinolone acetonide induced lipocortin I m-RNA expression in resting 3T3 fibroblasts. This is in agreement with data published by Pepinsky and Wallner (8,21) on glucocorticoid-induced lipocortin I expression in the human lymphoma cell line U937, however contrary to data published by Brönnegard et al. who could not find such an effect using human skin fibroblasts, peripheral lymphocytes, pulmonary alveolar macrophages and HeLa S3 cells (24). In confluent Swiss 3T3 cells the glucocorticoid-induced lipocortin I m-RNA expression was accompanied by a strong stimulation of cell proliferation. Both effects were

shown to depend on the presence of serum, indicating that an as yet unidentified serum factor may act synergistically with glucocorticoids on both lipocortin I expression and cell proliferation. It has been indeed suggested that the promoting effect of glucocorticoids on fibroblast proliferation may be related to their ability to synergise with fibroblast growth factor (23). Serum has been shown to stimulate PLA_2 activity and prostaglandin synthesis in Swiss 3T3 fibroblasts (19). Moreover, the release of arachidonic acid via PLA_2-catalyzed phospholipid degradation and the subsequent formation of eicosanoide was found to be essential for the induction of cell proliferation in BALB/c fibroblasts (22). If lipocortin acts as a cellular PLA_2 inhibitor, one would assume, therefore, that the lipocortin I expression observed upon FA treatment would result in an inhibition of arachidonic acid metabolism and, consequently, in a decrease of Swiss 3T3 fibroblast proliferation. Instead of, an increase of the proliferation rate was observed. A possible explanation would be that in Swiss 3T3 cells glucocorticoid-induced proliferation is not dependent on cellular arachidonic acid metabolism. Indeed, indomethacin and ETYA, two potent inhibitors of arachidonic acid metabolism, did not inhibit serum- or glucocorticoid-induced proliferation of 3T3-cells. Moreover, both agents were not able to inhibit glucocorticoid-induced lipocortin mRNA expression. These results indicate that the stimulatory effect of serum and glucocorticoid on the proliferation of quiescent 3T3 fibroblasts does not depend on eicosanoid synthesis. This would explain the inability of glucocorticoids to inhibit the growth of 3T3 cells. Whether there is a causal relationship between lipocortin I-gene expression and the growth promoting activity of glucocorticoids in these cells remains to be elucidated.

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