One of the mechanisms proposed to explain the anti-inflammatory activity of sodium salicylate (NaSal) is based, at least in part, on its ability to inhibit nuclear factor-κB activation and inhibition of nuclear factor-κB-dependent gene expression. On the other hand, little is known about the ability of NaSal to activate gene expression. By differential display reverse transcription polymerase chain reaction, we identified several genes that are modulated upon treatment of mouse fibroblasts with NaSal. From the various cDNA fragments recovered from autoradiograms, we found that NaSal can increase the levels of mRNA for biglycan, the mouse homologue of the human eIF-3 p47 unit, and immunophilin FKBP51. NaSal-induced expression of these genes was time- and dose-dependent. Moreover, FKBP51 gene expression was augmented in vivo, in mice treated orally or intraperitoneally with NaSal. We also found that treating cells with NaSal can inhibit the expression of the p34^cdc2 kinase. The impact this inhibition on cell cycle was evaluated by measuring the content of DNA during the cell cycle. Treatment of cells with NaSal led to a G_{2}/M arrest. By investigating the signaling events that regulate the expression of these genes and their biological activities, we can contribute to the understanding of the mechanism of NaSal.

Aspirin and salicylic acid constitute some of the most widely prescribed nonsteroidal anti-inflammatory drugs in the world (1). Diseases in which NSAIDs can mitigate symptoms and the inflammatory process include some joint diseases, such as rheumatoid arthritis and osteoarthritis. These drugs can also retard the progression of Alzheimer’s disease (2, 3).

Several mechanisms have been proposed in order to explain the ability of NSAIDs to act as anti-inflammatory regulators, especially those ascribed to aspirin and its metabolite, sodium salicylate (NaSal). Vane (4), in his classical work, proposed that the therapeutic effects of aspirin could be ascribed to its ability to inhibit prostaglandin biosynthesis, probably via a direct inhibition of cyclooxygenases. Although only aspirin, and not NaSal, effectively inhibits the biosynthesis of cyclooxygenase isozymes in a dose-dependent manner, both compounds are potent anti-inflammatory agents (5, 6). It has also been proposed that some effects of aspirin and NaSal that are apparently independent of prostaglandin biosynthesis are most likely due to the capacity of these drugs to insert themselves into the lipid bilayer of plasma membranes, where they disrupt signaling events and protein-protein interactions (7). Salicylates can also uncouple oxidative phosphorylation leading to ATP catabolism, diminishing intracellular ATP concentrations and, consequently, releasing micromolar amounts of adenosine, an autacoid with potent anti-inflammatory properties, into extra cellular fluids (8). At the molecular level, a more well-defined action of NaSal and aspirin is the inhibition of NF-κB activation (9). NF-κB is known as a transcription factor that mediates the expression of a variety of genes that regulate the inflammatory response, including several cytokines and adhesion molecules (10, 11). A possible pathway responsible for the inhibition of NF-κB activation by NaSal appears to be related to its ability to activate p38 mitogen-activated protein kinase. Activation of p38 mitogen-activated protein kinase will lead to the inhibition of IκB degradation and consequent inhibition of NF-κB activation (12). Also, inhibition of RSK2 kinase by NaSal leads to the inhibition of NF-κB-dependent gene expression (13). Furthermore, the ability of NaSal to block NF-κB activation can be attributed to the inhibition of the kinase activity of IκB kinase-β and its consequences, inhibition of IκB phosphorylation and degradation and subsequent translocation of active NF-κB to the nucleus (14).

So far, a number of genes of which the expression is affected by NaSal at suprapharmacological or pharmacological concentrations have been identified. Aspirin or NaSal can partially or completely inhibit the induced expression of a large number of genes, such as those for iNOS (15), adhesion molecules (16), TF (tissue factor) (17), some chemokines (18), apolipoprotein A (19), prostaglandin H synthase (20), and several cytokines (21, 22). Interestingly, a review of the literature shows a few genes that are induced by NaSal. Cytochrome P4502E1 in rat livers (23) and hsp70 in L929 cells (24) are the only examples of genes of which the expression levels are up-regulated by NaSal. The precise signaling pathway(s) responsible for NaSal-induced gene expression is not so well understood. Taking advantage of
differential display RT-PCR (25), we decided to search for new genes, the expression of which can be modulated by NaSal and/or TNF. Here, we demonstrate that genes such as Biglycan and FKBP51 have their expression augmented in cells treated with NaSal, whereas the expression of p34\(^{cdk}\) kinase is diminished in NaSal-treated cells. The function of the proteins encoded by these genes appears to be related to the anti-inflammatory action of NaSal, and understanding the mechanism by which NaSal modulates the expression of these genes will contribute to our understanding of its mechanism of action.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments—**Immortalized MEFs derived from sv129 mouse embryos were a gift from Drs. Charles Weissmann and Yi-Li-Yang (formerly at the Institute of Molecular Biology I, University of Zurich, Zurich, Switzerland). MEFs were cultured at 37 °C with 5% CO\(_2\) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, gentamicin (40 \(\mu\)g/ml), and nonessential amino acids. Cells were treated with NaSal (Sigma) (20 \(\mu\)M for 6 h) and/or TNF (R&D Systems) (20 \(\mu\)g/ml for 4 h).

**Mice and Administration of NaSal—**Mice (pure sv129 background) were maintained in a sterile atmosphere with autoclaved food and acidified water. Four- to 8-week-old male mice were killed either by cervical dislocation or intraperitoneally with 400 \(\mu\)g/kg NaSal. Control mice were treated with an equivalent volume of the vehicle. Animals were anesthetized with trichloroethylene vapor. Mice were treated orally or intraperitoneally with 400 \(\mu\)g/kg NaSal, whereas the expression of p34\(^{cdk}\) kinase was determined by Western blot analysis using an antibody specific for the p34\(^{cdk}\) protein.

**RNA Isolation and DD-RT-PCR—**Total RNA was isolated from confluent MEFs or from organs using TRIZOL (Life Technologies, Inc.) according to the manufacturer's instructions. Differential display was performed following the protocol of Liang and Pardee (25). After DNase I treatment, 200 ng of total RNA were mixed with one of the four anchored oligo(dT) primers at a final concentration of 2.5 \(\mu\)M each. Amplification products were separated by electrophoresis in a 8% polyacrylamide gel containing 8M urea and visualized by autoradiography. Bands of interest were excised from the gel, eluted in deionized water, and cloned. At least two clones from each ligation were sequenced, yielding a total of 94 clones with inserts of at least 100 nucleotides.

**Northern Blot Analysis and cDNA Probes—**Total RNA (200 ng) in the presence of anchored oligoT11VN. cDNA was used as template for PCR in the presence of the same anchored primer and one of the 10-nucleotide-long random primers (brackets labeled 13, 14 or 15). Radioactive PCR products were fractionated through a 6% denaturing polyacrylamide gel and exposed to x-ray film.**

**RESULTS**

**Differential Display RT-PCR and the Identification of NaSal- and/or TNF-modulated Genes—**A total of 44 PCRs were carried out in the presence of \([\alpha-\(^{32}\)P]dCTP by using one of the four anchored dT11VN primers in combination with one of three random 10-mers. On average, each lane in a differential display gel yielded about 80–100 distinguishable bands (Fig. 1). Fifty-nine bands representing cDNAs differentially expressed were excised and cloned. At least two clones from each ligation were sequenced, yielding a total of 94 clones with different inserts that were used for reverse Northern blot analysis. The size of cloned cDNA fragments ranged from 147 to 613 base pairs. The vast majority of clones were found to contain both random and anchored primers at their 5’ and 3’ ends, respectively. In a few clones, we found either the anchored or the random primers at both ends. The nucleotide sequences of the cloned cDNA fragments were compared against the nonredundant and expressed sequence tag data bases available on the GenBankTM sequence database.

**FIG. 1.** Representative DD-RT-PCR gel and band pattern from mRNA derived from control cells or from cells treated with NaSal, PCR, and NaSal plus TNF-stimulated MEFs. Cells were left untreated (c) or treated with NaSal (20 \(\mu\)M for 6 h) (n), with TNF (20 \(\mu\)g/ml for 4 h) (t), or with NaSal plus TNF (nt). The cDNA first strand was obtained by reverse transcriptase reaction using DNA-free total RNA (200 ng) in the presence of anchored oligoT11VN. cDNA was used as template for PCR in the presence of the same anchored primer and one of the 10-nucleotide-long random primers (brackets labeled 13, 14 or 15). Radioactive PCR products were fractionated through a 6% denaturing polyacrylamide gel and exposed to x-ray film. **Numbered arrowheads** indicate some of the cloned fragments.
network services. Of the 94 distinct clones that were generated, 40 were found to be identical to known mouse genes, 20 had significant similarity to rat or human genes, 15 were similar to expressed sequence tags (either mouse or human expressed sequence tags), and 19 showed nonsignificant homology with the sequences deposited in GenBank™. A summary of the clones of which the differential expression was confirmed by Northern blot analysis is given in Table I.

Evaluation of Differential Gene Expression upon Stimulation with NaSal and/or TNF—As a positive control for the stimulation of gene expression by TNF, we investigated the expression of KC chemokine and TSG-14 genes. Both are known to be up-regulated by TNF with functional NF-κB sites on their promoter region (27, 28). The mRNA steady-state level of both KC (Fig. 2) and TSG-14 (not shown) was elevated after TNF treatment but not after NaSal treatment. Furthermore, the TNF-induced expression of KC and TSG-14 was not impaired when the cells were co-stimulated with NaSal.

We next determined the steady-state levels of mRNA for other cDNAs identified by the DD-RT-PCR. As shown in Fig. 2, when MEFs were stimulated with NaSal, we detected an increase in the steady-state level of mRNA for biglycan, eIF-3p47 homologue, FKBP51, and es64. The es64 gene was also induced by TNF, but the combined TNF/NaSal treatment appears to have a synergistic effect.

The clone 14VC221, corresponding to p34cdc2 kinase, was chosen from the DD-RT-PCR because it appeared to be inhibited by NaSal. Indeed, when we performed Northern blot analysis using total RNA from untreated control cells or from cells treated with NaSal, TNF, or both together, we observed a weaker signal for the p34cdc2 kinase mRNA in lanes corresponding to cells exposed to NaSal (Fig. 2).

We next decided to further characterize the modulatory effect of NaSal on the expression of the above-mentioned genes, and because very little is known about stimulation of gene expression by NaSal, we gave priority to those genes of which the mRNA levels were up-regulated in the presence of the drug. First, we tested whether the up-regulation of FKBP51 observed in NaSal-treated MEFs could be reproduced in vivo, by measuring its mRNA level in organs of NaSal-treated mice. Northern blot analysis of spleen-derived total RNA from orally (Fig. 3A) or via intraperitoneal injection (Fig. 3B) using the 15VC462 clone as probe revealed that, indeed, FKBP51 mRNA is augmented in NaSal-treated mice. In orally treated mice, the peak of FKBP51 mRNA occurs between 4 and 6 h after treatment, whereas in mice injected intraperitoneally, the peak of induction occurs at 3 h postinjection (Fig. 3). Neither oral nor intraperitoneal treatment with NaSal led to the augmented expression of the FKBP12 gene.

We also determined the kinetics of biglycan and eIF-3 p47 homologue induction in MEFs treated with NaSal. As shown in Fig. 4, whereas no basal expression of biglycan could be detected, untreated cells had a measurable level of eIF-3 p47 mRNA, and the peak of mRNA steady-state levels for both biglycan and eIF-3 p47 homologue were obtained after 9 h of

### Table I

| Identification number | Clone name | Primer combination | Fragment size (base pairs) | Homology | Sequence identity |
|----------------------|------------|--------------------|---------------------------|----------|------------------|
| 1                    | 14A228     | 14 × T11VA         | 288                       | Mus musculus p34/cdc2 kinase (gb/U58633) | 286 /289 (98%) |
| 2                    | 14VC117    | 14 × T11VC         | 313                       | M. musculus p34/cdc2 kinase (gb/U58633) | 285 /299 (95%) |
| 3                    | 15A521     | 15 × T11VA         | 183                       | M. musculus es64 (homologous to human StAR protein) (gb/U36220) | 183 /183 (100%) |
| 4                    | 15VC677    | 15 × T11VC         | 294                       | M. musculus FK506-binding protein p51 (gb/U36220) | 294 /294 (100%) |
| 5                    | 13G418     | 13 × T11VG         | 613                       | Homo sapiens eucharistic initiation translation factor eIF-3/p47 subunit (gb/U94855) | 407 /465 (87%) |

* Corresponding to the bands assigned in Fig. 1.

* Only significant stretches of matching nucleotides between the query and subject sequences were analyzed.
stimulation (Fig. 4A). Co-stimulation of MEFs with NaSal and TNF for either 4 or 8 h did not alter the mRNA levels of biglycan or eIF-3 p47 homologue (Fig. 4B).

Reduced Levels of the p34<sup>cdc2</sup> Kinase mRNA in NaSal-treated Cells Led to Cell Cycle Arrest at the G<sub>2</sub>/M Transition—In order to evaluate the physiology of the inhibitory effect of NaSal on p34<sup>cdc2</sup> kinase gene expression, we compared, by FACS analysis, the percentage of cells during the various phases of the cell cycle in cultures that were treated with NaSal and their control counterparts. We first determined, by Northern blot analysis, that maximum reduction in p34<sup>cdc2</sup> kinase mRNA level occurred in cells treated with 20 μM NaSal for 9–12 h (Fig. 5A). However, at these time points, a significant amount of “dying” cells could be observed, as also pointed out by the reduced levels of GAPDH as compared with earlier points, regardless of our effort in accurately loading the gels. Therefore, we decided to perform cell cycle analysis in cells treated with NaSal for 6 h. Fig. 5B shows one representative of two experiments in which we observed that, in NaSal-treated cultures, 7.68% of cells were at the G<sub>2</sub>/M transition phase, as compared with 3.69% of cells at the same transition phase in control cultures of cells. In Fig. 5C, we show the average of a duplicate experiment that confirms the increased percentage of cells at the G<sub>2</sub>/M transition phase in NaSal-treated cultures.

**DISCUSSION**

Among the NSAIDs, acetylsalicylic acid, better known as aspirin, and other aspirin-like drugs are the most widely prescribed (1) and have been used clinically for more than a century. Nevertheless, only recently have we gained some information on the molecular aspects of the mechanism by which these drugs can modulate the expression of inflammation-related genes. From the seminal work of Vane (4), suggesting that inhibition of prostaglandin biosynthesis is the major anti-inflammatory action of these drugs, we now know that NaSal can also inhibit the activation of p38 kinase, a key event in the inhibition of NF-κB activation (12). What is less clear is how NaSal and other aspirin-like compounds can activate gene expression. Thus far, only a few genes, such as the hepatic cytochrome p4502E1 (23, 29), are known to be induced by NaSal. It has also been suggested that some NSAIDs can induce the expression of the HSP70 gene in human monocytes (30) and in L929 cells (24). However, in HeLa cells, NaSal did not activate HSP70 gene transcription. In these cells, it appears that the transient acquisition of DNA binding activity by
the transcription factor HSF1 is not sufficient to drive gene expression (31).

Using the differential display technique (25), we identified a series of genes of which the expression is modulated by NaSal on mouse embryonic fibroblast.

Among the genes of which the mRNA level was found to be diminished in NaSal-treated cells, we identified the cycle-dependent \( p34^{cd2} \) kinase (32). We observed that upon treatment of mouse embryonic fibroblasts with NaSal, \( p34^{cd2} \) kinase mRNA decreased in a dose- and time-dependent manner, leading to G2/M arrest in mouse cells. It was previously reported that aspirin or indomethacin reduces the levels of \( p34^{cd2} \) kinase in HT-29 colon adenocarcinoma cells, reducing the proportion in the S phase (33). From the technical point of view, the fact that we cloned three independent cDNA fragments corresponding to \( p34^{cd2} \) kinase, all of them with a similar pattern in the gels, provides an excellent internal control for the reliability and reproducibility of the differential display, showing a correlation of this finding with the data already reported in the literature.

The identification of at least four genes up-regulated by NaSal opens a new field of investigation of NaSal mode of molecular action. These genes, which have not yet been identified as being responsive to NaSal, encode a small chondroitin/dermatan sulfate proteoglycan biglycan (PG-D) (36, 37), FK506-binding protein p51 (FKBP51) (38), es64 (homologous to the human STAR protein) (39), and a putative eukaryotic translation initiation factor 3 (eIF-3) p47 subunit (40).

Of these genes, only the Biglycan gene has had its promoter structure identified and functionally characterized. In humans, its induction appears to be controlled via protein kinase A and interaction of SP3- and SP1-like factors (41). More recently, a binding site for the transcription factor cKrox, originally found as regulator of type I collagen gene expression (42, 43), was also found at position –248 to –230 of the human biglycan gene (44). In the mouse, the expression of the biglycan gene appears to be regulated by SP-1, AP-1, and AP-2 transcription factors with low levels of expression in skin (37).

Based on these observations, and principally on the functional characterization of the promoter region of the other NaSal-stimulated genes described herein, it will be possible to investigate the molecular events in NaSal-triggered signal transduction. Further characterization of cis-elements and trans-acting factors that regulate the expression of other NaSal-stimulated genes will greatly contribute to our understanding of the molecular aspects of the mode of action of NSAIDs.

The characterization of the induced expression of the FKBP51 gene by NaSal and other NSAIDs, such as aspirin or indomethacin, could be of great importance for the understanding of the mode of action of aspirin. The FKBP51 gene was initially identified as a gene regulated by glucocorticoids in murine thymocytes (45) and subsequently characterized as member of family of intracellular receptors for immune suppressant drugs, such as FK506 and rapamycin (38). Moreover, FKBP51 was also identified by DD-RT-PCR as being probably involved in the adipocyte differentiation (46). Interestingly, NaSal was a potent inducer of FKBP51 both in vitro and in vivo, but it failed to induce the expression of the FKBP12 gene, the intracellular receptor for cyclosporin.

This differential induction of the two genes provides an important piece of evidence for a specific signaling cascade triggered by NaSal; a comparison of the promoter structure of these genes will help in the elucidation of NaSal mode action and could have an important impact in the administration of immune suppressant drugs.

Acknowledgments—We thank Elisângela Monteiro and Anna C. M. Salim (Laboratory of Cancer Genetics, Ludwig Institute for Cancer Research, São Paulo, Brazil) for the sequencing data. Also, we are grateful to Dr. M. M. Brentani, M. A. Koique, and Rose Roela from the Department of Radiobiology (Faculdade de Medicina, Universidade de São Paulo) for helping with the FACS data. We thank all members of our laboratory for helpful discussions.

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