In Murine 3T3 Fibroblasts, Different Second Messenger Pathways Resulting in the Induction of NO Synthase II (iNOS) Converge in the Activation of Transcription Factor NF-κB*

(Received for publication, September 18, 1995, and in revised form, November 29, 1995)

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Transcription factor NF-κB is essential for the induction of nitric oxide synthase (NOS) II (iNOS) by bacterial lipopolysaccharide in murine macrophages (Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994) J. Biol. Chem. 269, 4705-4708). In 3T3 fibroblasts, agents other than cytokines are efficacious inducers of NOS II expression. In addition to cytokines such as interferon-γ or tumor necrosis factor-α, protein kinase C-stimulating agents such as tetradecanoylphorbol-13-acetate, or cyclic AMP-elevating agents such as forskolin and 8-bromo-cAMP markedly increased NOS II mRNA (measured by S1 nuclease and reverse transcriptase and RNase protection analyses), NOS II protein (determined by Western blotting), and NOS activity (measured by chemiluminescence detection of NO2). Transforming growth factor-β1 (which is an inhibitor of NOS II induction in other cell types) potentiated NOS II mRNA expression produced by all inducing agents listed, whereas dexamethasone, pyrrolidine dithiocarbamate and 3,4-dichloroisocoumarin (inhibitors of NF-κB activation) suppressed NOS II mRNA induction in response to all stimulants. In electrophoretic mobility shift assays, nuclear protein extracts from 3T3 cells stimulated with any of the inducing agents significantly slowed the migration of an NF-κB-binding oligonucleotide, whereas nuclear extracts from untreated control cells did not. These experiments indicate that NF-κB is the key control element for the induction of NOS II in response to at least three different second messenger pathways in 3T3 cells.

Nitric oxide (NO) is a short-lived bioactive molecule particiating in the physiology and/or pathophysiology of many organ systems (1). The expression of the inducible isoform of nitric oxide synthase (NOS II or iNOS) is regulated mainly at the transcriptional level (2). Inflammatory stimuli such as bacterial lipopolysaccharide (LPS) and cytokines induce the expression of this enzyme in many cell types. Interestingly, in some cells, agents other than cytokines are efficacious inducers of NOS II expression. For example, in rat mesangial cells, cAMP-elevating agents stimulate NOS II expression (3). Phorbol ester induction of NOS II has been reported for rat peritoneal macrophages (4). In murine BALB 3T3 fibroblasts, NOS II is expressed in response to forskolin, dibutyryl cAMP, or tetradecanoylphorbol-13-acetate (TPA) (5).

Analyses of the deduced murine NOS II promoter (6-8) have revealed the presence of numerous consensus sequences for the binding of transcription factors. Of these potentially relevant transcription factors, nuclear factor-κB (NF-κB) (6, 9) and interferon regulatory factor (10, 11) have been shown to be functionally important for NOS II induction. The molecular mechanisms utilized by other second messenger pathways are still unclear. In rat mesangial cells, the inhibitor of NF-κB activation, pyrrolidine dithiocarbamate (PDTc), blocked NOS II expression induced by interleukin-1β (IL-1β), but not the expression stimulated by 8-bromo-cAMP, suggesting two different induction pathways (12).

In the current study, we attempted to induce NOS II expression in 3T3 fibroblasts via four different second messenger pathways, namely receptor tyrosine kinase, protein kinase C, protein kinase A, and protein kinase G. We characterized the induction processes with modulators of NOS II induction such as transforming growth factor-β1 (TGF-β1), dexamethasone, PDTc, and 3,4-dichloroisocoumarin (DCI). The experiments indicate that all NOS II-inducing second messenger pathways are modulated in the same way and all converge in the activation of NF-κB as an essential transcription factor.

MATERIALS AND METHODS

Reagents—Mouse INF-γ, human TNF-α, and human TGF-β1 were purchased from Genzyme. LPS (Escherichia coli 026:B6), PDTc, DCI, dexamethasone, forskolin, 8-bromo-cAMP, 8-bromo-GMP, isobutylmethylxanthine (IBMX) and TPA were purchased from Sigma. Isotopes were obtained from Amersham Corp. Restrictions enzymes, polyomavirus, Taq polymerase, S1 nuclease, T3 RNA polymerase, dNTPs, and oligo(dT) primer were purchased from Pharmacia Biotech Inc. RNase ONE™ was obtained from Promega. Superscript reverse transcriptase and DNase I were obtained from Life Technologies, Inc.

Cell Culture—Murine BALB 3T3 fibroblasts and RAW 264.7 macrophages (both ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. For induction, confluent 3T3 cells were cultured for 16 h in DMEM with only 0.5% fetal calf serum and then incubated for 3-18 h (in DMEM with 0.5% fetal calf serum) with one of the following agents: INF-γ (100 units/ml), TNF-α (10 ng/ml), LPS (1 μg/ml), TPA (50 ng/ml), forskolin (100 μm), 8-bromo-
cAMP (1 mM), IBMX (250 μM), or 8-bromo-cGMP (1 mM). In some experiments the following modulators of NOS II induction were added with one of the inducing agents: dexamethasone (5 μM), TGF-β1 (12 ng/ml), PDTC (100 μM), or DCP (50 μM).

NOS II Protein Preparation and Western Blotting—3T3 cells (un-treated or induced for 18 h with TNF-α, 10 ng/ml; LPS, 1 μg/ml; TPA, 50 ng/ml; IFN-γ, 100 units/ml) were washed by previously for brain tissue or endothelial cells (13, 14). Homogenates were centrifuged at 100,000 × g for 1 h, and the soluble (cytosolic) fraction was partially purified on 2.5'-ADP-Sepharose (13, 14). The eluates from the affinity columns were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% gels) (15). The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell), electroblotted (Bio-Rad). All subsequent steps were performed at room temperature. The blots were blocked in Blotto (3% non-fat dried milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 45 min. The blots were incubated for 1 h with a monoclonal anti-NOS II antibody (1 μg/ml, Transduction Laboratories, Lexington, KY) in Tris-buffered saline/Tween (TBS/T: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 50 μl filter gelatin. The blots were washed twice with TBS/T (7 min each) and then incubated for 30 min with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2000 in TBS/T with 50 μl filter gelatin. The blots were washed three times (5 min each) in TBS/T, followed by one wash (5 min) in TBS alone. The immunocomplexes were developed using an enhanced horseradish peroxidase-luminol chemiluminescence reaction (DuPont NEN) according to the manufacturer’s instructions.

Cloning of a Murine NOS II and a Murine β-Actin cDNA Fragment—Total RNA was isolated by guanidinium isothiocyanate/phenol/chloroform extraction (16) from RAW 264.7 cells induced with 1 μM LPS for 16 h. Two μg of this RNA were annealed with 0.5 μl of oligo(dT) primer (Pharmacia) and reverse-transcribed with Superscript reverse transcriptase (Life Technologies, Inc.) following the manufacturer’s instructions. Reverse transcriptase-generated cDNA encoding for murine NOS II and murine β-actin were amplified using PCR. Oligonucleotide primers for NOS II and β-actin were: GACAAGAGCTGCTCCCCC (sense), and GCTGGGACTCATGGAGCG (antisense); GTGGGCCGCTCTAGA and CTTTGGATCCGCG (antisense), respectively. They generated PCR fragments corresponding to the murine NOS II cDNA (17) (positions 2612–3170) and murine β-actin (18) (positions 25–564). PCR was performed in 100 μl of Taq polymerase buffer (Pharmacia), containing 0.2 mM dNTPs, 1.5 mM MgCl2, 2 units of Taq polymerase, 50 pmol oligonucleotide primers, and reverse transcriptase products (0.10 of the reverse transcriptase reaction). After a initial denaturation step of 95°C for 5 min, 30 cycles were performed (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C). The final extension period at 72°C was 10 min. Amplified cDNA fragments (NOS II, 559 base pairs; β-actin, 540 base pairs) were done into the EcoRV site of pCR-Script (Stratagene) using the Sure Clone ligation kit (Pharmacia), generating the cDNA clones pCR_NOS II mouse and pCR_β-actin mouse of the cloned PCR products were determined from plasmid templates using the dyeoxy chain termination method with the T7 sequencing kit (Pharmacia).

Preparation of DNA and Antisense RNA Probes—To generate radiolabeled DNA probes for S1 nuclease protection analysis, the cDNA clones pCR_NOS II mouse and pCR_β-actin mouse were restricted with NcoI or BglII, dephosphorylated (with calf intestinal alkaline phosphatase, Boehringer Mannheim), extracted with phenol/chloroform, and concentrated by ethanol precipitation. Fifty to one hundred ng of this DNA were labeled with [γ-32P]ATP using polynucleotide kinase (Pharmacia). The radiolabeled DNA was separated from unincorporated radioactivity using Nuc Trap probe purification columns (Stratagene). Radiolabeled antisense RNA probes for RNase protection assays, the cDNA clones pCR_NOS II mouse and pCR_β-actin mouse were linearized with NcoI or BstEII, extracted with phenol/chloroform, and concentrated by ethanol precipitation. One half of this DNA was in vitro transcribed using T3 RNA polymerase (Pharmacia) and [γ-32P]UTP. After a 1-h incubation, the transcription reaction was degraded with DNasel for 15 min. The radiolabeled RNA was purified using Nuc Trap probe purification columns (Stratagene).

S1 Nuclease Protection Analyses and RNase Protection Analyses—S1 nuclease protection analyses were performed as described (19, 20). Briefly, after a denaturation step at 85°C for 30 min, 20 μg of total RNA isolated by the guanidinium isothiocyanate/phenol/chloroform-extraction method (16) were hybridized at 52°C for 16 h with 75,000 cpm labeled NOS II DNA probe and 30,000 cpm labeled β-actin DNA probe in hybridization buffer (40 μM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide) in a volume of 30 μl. The S1 nuclease digestion was started by adding 310 μl of digesting buffer (280 μM NaCl, 4.5 mM ZnCl2, 0.05 mM denatured salmon sperm DNA, and 300 units/ml S1 nuclease). After 20 min at 37°C, the reaction was stopped by adding 65 μl of stop-buffer (2.5 M NH4-acetate, 50 mM EDTA), followed by a phenol/chloroform extraction. The reaction products were precipitated by ethanol precipitation on ice. The precipitates were redissolved in electrophoresis in denaturing urea-polyacrylamide gels (8% urea, 6% polyacrylamide gel electrophoresis). The electrophoresis buffer was 1 x TBE (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA). The gels were electrophoresed for 2–3 h, dried, and exposed to x-ray films. The protected DNA fragments of NOS II and β-actin were 380 and 150 nucleotides, respectively. RNase protection assays were performed with RNase ONE™ according to the manufacturer’s instructions (Promega). Briefly, following denaturation, 20 μg of total RNA (prepared as described above) were hybridized with 100,000 cpm labeled NOS II anti-sense RNA probe and 10,000 cpm labeled β-actin antisense RNA probe at 51°C for 16 h in a volume of 30 μl. Then the mixture was digested with 5 units of RNase ONE™ for 1 h at room temperature in 300 μl. The reaction was stopped with 1% SDS, and the samples were concentrated and electrophoresed as described for the S1 nuclease protection analyses. The protected RNA fragments of NOS II and β-actin were 184 and 108 nucleotides, respectively.

Electrophoretic Mobility Shift Assay (EMSAs)—NF-κB binding activity in the nuclei of control 3T3 fibroblast- or RAW 264.7 cells, or cells induced with the NOS II-inducing agent (as described above) were determined by EMSA using the Promega gel shift assay system. Nuclear proteins were extracted from the cells by detergent lysis (21). Ten μg of nuclear protein were incubated with 17.5 fmoles 32P-labeled double-stranded oligonucleotide containing a motif for NF-κB binding (5′-AGTTGAGGGGACTTTCCCAGGC-3′). In some experiments, 1.75 pmol of an oligonucleotide with the putative NF-κB binding sequence of the murine NOS II promoter (5′-CAAGTGGG-GACTCTCCGTTTGG-3′) were added. The DNA-protein complexes were analyzed on 5% polyacrylamide gels (electrophoresis buffer: 6.7 mM Tris/HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA), dried, and autoradiographed.

Measurement of NO Production by Chemiluminescence—Confluent 3T3 fibroblasts were cultured for 18 h in DMEM containing 10% fetal bovine serum. Control cells received no additions to the medium; other cells were incubated with LPS (1 μg/ml), TNF-α (10 ng/ml), TPA (50 ng/ml), or forskolin (100 μM). After 18 h, the cell supernatants were collected and aliquots were deproteinized with 2 volumes of ethanol. Following centrifugation, 200 μl of the supernatant were injected into a chemiluminescence chamber containing 100 μl KI in 10 mM sulfuric acid. This strong reducing environment converts NO2 (and nitrosyl compounds) back to NO. A constant stream of N2 gas carried the NO into a nitric oxide analyzer (Sievers, Boulder, CO) where the NO was reacted with ozone, resulting in the emission of light. The light emission is proportional to the NO formed; standard amounts of NO2 were used for calibration.

RESULTS AND DISCUSSION

Stimulation of Different Second Messenger Pathways Induced NOS II mRNA Expression—In 3T3 cells, NOS II mRNA was markedly induced with IFN-γ (100 units/ml) or TNF-α (10 ng/ml) (Fig. 1). NOS II expression was also enhanced with TPA (50 ng/ml) or the cAMP-elevating agents forskolin (100 μM) or 8-bromo-cAMP (1 mM) (Fig. 2). In contrast, 8-bromo-cGMP (1 mM) was ineffective as a stimulator of NOS II induction (Fig. 2). The phosphodiesterase inhibitor IBMX (250 μM) also produced a marked induction of NOS II mRNA in 3T3 cells (Fig. 3). This can be explained by the increase in cAMP, but not cGMP (cf. Fig. 2). LPS (up to 1 μg/ml) showed little efficacy in inducing NOS II mRNA (Fig. 3). Thus the stimulation of the receptor tyrosine kinase pathway (by IFN-γ, TNF-α, and possibly LPS), the stimulation of the protein kinase C pathway (by TPA), and the stimulation of the protein kinase A pathway (by forskolin, 8-bromo-cAMP, and IBMX) all induced the transcription of NOS II mRNA in 3T3 fibroblasts.

Because double protected bands for NOS II mRNA were seen in some of the S1 nuclease analyses, RNase protection assays were performed on the same RNAs (using antisense RNA probes derived from the same NOS II cDNA fragment).
Stimulation of Different Second Messenger Pathways in 3T3 Cells

- 3T3 fibroblasts respond to a variety of stimulants with different pathways.
- INF-γ alone is an effective NOS II inducer in 3T3 cells.
- The signal transduction pathways affecting NOS II expression vary considerably between cell types.
- INF-γ has little to no effect on NOS II induction by itself, but it potentiates cytokine induction.
- Other stimulators of the protein kinase C pathway alone can induce NOS II in RAW 264.7 macrophages.
- The signal transduction mechanisms seem to be cell-specific.

NF-κB and NOS II Induction in 3T3 Fibroblasts

NF-κB activation seems to inhibit NOS II induction in rat RINm5F insulinoma cells. Thus, the NOS II-inducing mechanisms may either represent a low constitutive expression of this isoform or an autocrine/paracrine induction of these cells by endogenous cytokines.

In 3T3 cells, INF-γ alone produced a marked induction of NOS II mRNA (Fig. 1). There is controversy as to whether INF-γ alone can induce NOS II in RAW 264.7 macrophages. While this has been described by some authors, others only see an effect of INF-γ in the presence of LPS (6, 7).

In the current experiments, polymyxin B (10 μg/ml), an inhibitor of the induction of murine cells by LPS (23), did not prevent the NOS II-inducing action of INF-γ (100 units/ml), TNF-α (10 ng/ml), TPA (50 ng/ml), or forskolin (100 μM) (n = 4, data not shown), suggesting that INF-γ alone is an effective NOS II inducer in 3T3 cells.

The signal transduction pathways effective in inducing NOS II expression vary considerably between cell types. In many cells, stimulation of the protein kinase C pathway has little to no effect on NOS II induction by itself, but it potentiates cytokine induction.

In 3T3 fibroblasts, it is an efficacious inducing pathway by itself. Stimulators of the protein kinase A pathway alone have been shown to promote NOS II expression in vascular smooth muscle cells and rat mesangial cells (12, 27, 28). On the other hand, protein kinase A activation seems to inhibit NOS II induction in rat RINm5F insulinoma cells (26). Thus, the NOS II-inducing mechanisms seem to be cell-specific, and the stimulation pattern observed in the present study (Figs. 1–3) is unique to 3T3 cells.

Stimulation of Different Second Messenger Pathways in 3T3 Cells

- Stimulation of different second messenger pathways in 3T3 cells increased NOS II protein expression.
- Similar to the NOS II mRNA expression induced by various signal transduction pathways, expression of NOS II immunoreactive protein was stimulated by TNF-α (10 ng/ml), LPS (1 μg/ml), TPA (50 ng/ml), or forskolin (100 μM).

Non-induced 3T3 cells showed no NOS II immunoreactivity in Western blots (n = 3, not shown).

Stimulation of Different Second Messenger Pathways in 3T3
Cells Enhanced NO\textsubscript{2} Production—Incubation of 3T3 fibroblasts with TNF-\alpha, TPA, or forskolin markedly enhanced the NO\textsubscript{2} content in the supernatant of the cells (Fig. 5). LPS was a much weaker stimulant of 3T3 cell NO\textsubscript{2} production (Fig. 5). This indicates that NOS II protein and activity is also induced via the receptor tyrosine kinase, protein kinase C, and protein kinase A pathways.

Stimulation of Three Different Second Messenger Pathways in 3T3 Cells Induced Proteins with NF-\kappaB Binding Activity—NF-\kappaB is a multisubunit transcription factor that can rapidly activate the expression of genes involved in immune and acute phase responses (29). NF-\kappaB is composed mainly of proteins with molecular weights of 50 kDa (p50) and 65 kDa (p65). Both types of proteins share significant homology with the proto-oncogene c-rel (30–32). The proteins p50, p65, and c-Rel can interact with each other and, following activation, bind the NF-\kappaB response element as homo- or heterodimers (33) (consensus sequence: GGGRNNYYCC) (34). In its unstimulated form, NF-\kappaB is present in the cytosol bound to the inhibitory protein I-kB. After induction of cells by a variety of agents, NF-\kappaB is released from I-kB and translocated to the nucleus. Agents that have been described as NF-\kappaB activators include mitogens, cytokines, and LPS, TPA, and cAMP (29, 35). EMSA experiments shown in Fig. 6 demonstrated that nuclear extracts of untreated 3T3 cells contained low concentrations of proteins that bind an oligonucleotide containing the NF-\kappaB response element. Incubation of 3T3 cells either with TPA (50 ng/ml), TNF (10 ng/ml) or 8-bromo-cAMP (1 mM) markedly increased the NF-\kappaB binding activity (Fig. 6). In 3T3 fibroblasts, TNF-\alpha was the most efficacious inducer of NF-\kappaB binding activity tested. TPA and cAMP-elevating agents (8-bromo-cAMP or forskolin) were less efficacious in inducing NF-\kappaB binding activity; there were no significant differences in efficacy between these two classes of agents. This parallels the NOS II mRNA and NOS II protein expression as well as the NOS activity stimulated by these compounds. The protein-DNA interaction was totally prevented in all cases with an excess of unlabeled double-stranded oligonucleotide containing the NF-\kappaB site of the murine NOS II promoter (Fig. 6 and data not shown). These data suggest that, in 3T3 cells, the receptor tyrosine kinase pathway, the protein kinase A pathway, and the protein kinase C pathway stimulate the activation of transcription factor NF-\kappaB. While cytokines such as TNF-\alpha can activate NF-\kappaB in most cell types, there is a marked inter-cell variability for the protein kinase A and C pathways. For example, in murine RAW264.7 cells, neither the protein kinase A pathway nor the protein kinase C pathway are able to stimulate this transcription factor; they even inhibit NF-\kappaB-dependent reporter gene expression in response to LPS (36). In human J urkat T cells, the protein kinase C pathway, but not the protein kinase A pathway activates NF-\kappaB (37). Conversely, in murine J 774 macrophages, activators of protein kinase A are effective stimulators of NF-\kappaB, whereas protein kinase C activators failed to stimulate this transcription factor (38).

Effect of Dexamethasone on NOS II mRNA Expression—Glucocorticoids such as dexamethasone have been known for some years to inhibit cytokine induction of NOS II activity in various cell types (such as endothelial cells, macrophages, and smooth muscle cells (39–42)). More recently, this inhibition has also been demonstrated at the mRNA level in several cell types (5, 43, 44). In a recent communication, Kunz et al. (45) demonstrated in rat mesangial cells that dexamethasone prevented the induction of NOS II activity in response to IL-1\beta and dibutyryl cAMP. Interestingly, NOS II mRNA levels were only reduced when dibutyryl cAMP was used as the inducing agent, but not after IL-1\beta. Consequently, these authors postulated that dexamethasone acts at different levels, depending on the stimulus used to suppress NOS II induction in rat mesangial cells (45). In the current study we examined the effect of dexamethasone (5 \mu M) on NOS II mRNA expression in 3T3 cells. We found that the steroid was equally effective against inductions produced by LPS, TPA or IBMX (Fig. 3). Also the NOS II mRNA inductions in response to TNF-\alpha (10 ng/ml) or INF-\gamma (100 units/ml) were markedly inhibited by dexamethasone (5 \mu M) (n = 3, not shown).

Effect of TGF-\beta1 on NOS II mRNA Expression—TGF-\beta1 is an inhibitor of NOS II induction in mouse macrophages and rat vascular smooth muscle cells (42, 43, 46–48). On the other hand, in 3T3 cells and in bovine retinal pigmented epithelial cells, TGF-\beta1 has been described as a stimulator of cytokine-induced NOS II mRNA induction (43, 49). Also in the current experiments, TGF-\beta1 (2 ng/ml) potentiated NOS II mRNA production irrespective of the second messenger pathway used for induction (Fig. 7).

Inhibition of NF-\kappaB Activation Blocks NOS II mRNA Induction—The activation of NF-\kappaB can be blocked by thiol compounds such as PDTC or diethylthiocarbamate, which leave the DNA binding activity of other transcription factors (e.g.,
NF-κB and NOS II Induction in 3T3 Fibroblasts

Macrophages have been shown to prevent the induction of NOS II in LPS-induced murine macrophages (51) and rat alveolar macrophages (52). Eberhardt et al. (12) reported that PDTC inhibits the induction of NOS II expression in response to IL-1β, but not to dibutyryl cAMP. They concluded that in rat mesangial cells cAMP-stimulated NOS II expression is activated through a transcription factor different from NF-κB. In the current series of experiments in 3T3 cells, PDTC prevented the induction of the NOS II mRNA expression in response to all inducing compounds used (Fig. 8). Also DCI, a serine protease inhibitor, which blocks NF-κB activation by inhibiting proteolytic degradation of IκB (53), blocked (by over 90%) NOS II mRNA expression induced by INF-γ (100 units/ml), TNF-α (10 ng/ml), TPA (50 ng/ml), and forskolin (100 μM) (n = 3, data not shown). This confirms the results of our EMSA experiments and indicates that in 3T3 fibroblasts NF-κB is essential for NOS II induction in response to different second messengers. Interestingly, the inhibition of NOS II induction by dexamethasone (described above) is likely to reflect its ability to inactivate NF-κB (20).

In conclusion, our data demonstrate that in 3T3 cells at least three different signal transduction pathways can stimulate NOS II mRNA expression, namely the cytokine/receptor tyrosine kinase pathway, the cAMP/protein kinase A pathway, and the protein kinase C pathway. All these pathways seem to converge in the activation of the essential transcription factor NF-κB, which increases the transcription of the NOS II gene.

Acknowledgment—We greatly appreciate the technical help of Bärbel Hering with the cell culture.

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J. Biol. Chem. 1996, 271:6039-6044.
doi: 10.1074/jbc.271.11.6039

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