Inhibition of Translation Initiation Mediates the Anticancer Effect of the n-3 Polyunsaturated Fatty Acid Eicosapentaenoic Acid

Sangeetha S. Palakurthi, Rudolf Flückiger, Hussein Aktas, Arun K. Changolkar, Aliakbar Shahsafaei, Silvia Harneit, Ergin Kilic, and Jose A. Halperin

ABSTRACT

Eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid that is abundant in the fish-based diets of populations that exhibit a remarkably low incidence of cancer, exerts anticancer activity in vitro and in animal models of experimental cancer. Here we define the molecular basis for the anticancer effects of EPA. EPA inhibits cell division by inhibiting translation initiation. This is a consequence of the ability of EPA to release Ca\(^{2+}\) from intracellular stores while inhibiting their refilling via capacitative Ca\(^{2+}\) stores and thereby activation of protein kinase R. Protein kinase R phosphorylates and inhibits eukaryotic initiation factor 2\(\alpha\), resulting in inhibition of protein synthesis at the level of translation initiation, preferentially reducing the synthesis and expression of growth-regulatory proteins, including G1 cyclins, and causes cell cycle arrest in G\(_1\). In a KLN-205 squamous cell carcinoma mouse model, daily oral administration of EPA resulted in a significant reduction in tumor size and expression of cyclin D1 in the tumor tissues. Furthermore, EPA-treated tumors showed a significant increase in the proportion of diploid cells, indicative of cell cycle arrest in G\(_0\)-G\(_1\), and a significant reduction of malignant hypertetraploid cells. These results characterize EPA as a member of an emerging new class of anticancer compounds that inhibit translation initiation.

INTRODUCTION

The n-3 PUFA\(^3\) EPA inhibits the proliferation of cancer cells in vitro (1–3) and the growth and metastatic potential of tumors in animal models of experimental cancer (4–6). Although alterations of eicosanoid formation and free radical-mediated lipid peroxidation have been mentioned as possible explanations for these observations (7, 8), the molecular basis for the pharmacological anticancer activity of EPA has not been elucidated. Epidemiological evidence suggests that populations consuming diets rich in fish oils containing EPA have a remarkably low incidence of cancer in general and of breast and prostate cancer in particular (9–11). Defining this mechanism of anticancer activity of EPA is important for its potential impact not only on cancer therapy but also on cancer prevention. In this report, we define the molecular basis for the anticancer activity of EPA.

Recent work from our laboratory has established a distinct connection between partial depletion of intracellular Ca\(^{2+}\) stores and inhibition of both cell proliferation and tumor growth (12). Depletion of Ca\(^{2+}\) stores activates IFN-inducible PKR, which phosphorylates and thereby inhibits the translation initiation factor eIF2, resulting in inhibition of translation initiation, reduced synthesis and expression of G1 cyclins, and cell cycle arrest in G\(_1\) (13). Indeed, regulation of translation initiation plays a critical role in the control of cell growth and division (14) because translation of most growth-regulatory proteins and oncogenes is highly inefficient because of the presence of stable secondary structures in the 5′ UTR of their mRNAs (15, 16). The length and secondary structure of the 5′ UTR of a mRNA molecule is the most critical feature influencing its translation efficiency. A moderately long, unstructured 5′ UTR with low content of G and C bases seems to be optimal to ensure high translational efficiency. In contrast, long 5′ UTRs with a high GC content are a major barrier to translation because G and C bases tend to form bonds that stabilize the secondary structure of the 5′ UTR. Sequence analyses of a large number of vertebrate cDNAs have shown that mRNAs with complex, highly structured 5′ UTRs include a disproportional high number of proto-oncogenes such as the G1 cyclins, transcription and growth factors, cytokines, and other proteins critical in cell growth regulation. In contrast, mRNAs that encode housekeeping proteins rarely have highly structured GC-rich 5′ UTRs (14, 15, 17). These structural differences in the 5′ UTR of their mRNA explain why translation of growth-regulatory proteins including cyclins but not of housekeeping proteins is highly dependent on the activity of translation initiation factors such as eIF2 and eIF4E. The critical role of translation initiation in cell growth regulation is illustrated by the finding that activating mutations of some translation initiation factors cause malignant transformation. For example, malignant transformation of cells can be induced by the expression of a dominant-negative form of PKR, which regulates eIF2 activity (18) by a constitutively active, noninhibitable mutant of eIF2 (eIF2-\(\alpha\)51A; Refs. 19 and 20) or by the overexpression of eIF4E (21). In contrast, repression of eIF4E activity by overexpression of eIF4E binding proteins inhibits cell growth and can reverse a malignant phenotype (22–24). Furthermore, compounds such as clotrimazole that activate PKR and phosphorylate eIF2 by causing partial depletion of intracellular Ca\(^{2+}\) stores are potent inhibitors of cell growth (13) and tumor growth in experimental cancer models (12).

EPA is likely to induce partial depletion of intracellular Ca\(^{2+}\) stores because it releases Ca\(^{2+}\) from IP\(_3\)-sensitive Ca\(^{2+}\) pools (25) and inhibits store-dependent capacitative Ca\(^{2+}\) influx (26). This suggested to us that EPA might inhibit translation initiation and thereby inhibit synthesis and expression of cell growth-regulatory proteins. We report here that by releasing Ca\(^{2+}\) from intracellular stores while preventing their refilling via capacitative Ca\(^{2+}\) influx, EPA causes PKR-mediated phosphorylation of eIF2\(\alpha\), inhibition of translation initiation, and preferential inhibition of synthesis and expression of G1 cyclins with consequent cell cycle arrest in G\(_1\). In a mouse KLN-205 squamous cell carcinoma model, oral EPA administration significantly reduced tumor growth and the expression of cyclin D1 in tumor cells while causing cell cycle arrest in G\(_0\)-G\(_1\). This work defines EPA as an inhibitor of translation initiation, a new emerging class of drugs for cancer therapy.

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3 The abbreviations used are: PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; PKR, protein kinase R; eIF2\(\alpha\), eukaryotic initiation factor 2\(\alpha\); UTR, untranslated region; IP\(_3\), inositol triphosphate; bFGF, basic fibroblast growth factor; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Materials. EPA (Sigma Chemical Co., St. Louis, MO), 99% pure, was diluted in DMSO to a concentration of 60 mM, and aliquots were blanketed with nitrogen and stored at −80 °C. α-3 PUFAs concentrate containing 300 mg of EPA, 200 mg of docosahexaenoic acid, and 1 IU vitamin E/gelatin capsule.

Cell Culture and Transfection. NIH 3T3 cells were cultured in DMEM/10% calf serum/penicillin/streptomycin, except during the mitogenic assays and in experiments for synthesis and expression of G1 cyclins with EPA treatment, when the cells were in DMEM/0.1% calf serum/bFGF (5 ng/ml). NIH 3T3 cells were transfected with pBABE (vector alone), cotransfected pBABE either with eIF2α-51A or dominant-negative PKR (PKR-K296), and purumycin-resistant stable colonies were constructed and maintained in DMEM/10% calf serum with 2.5 μg/ml purumycin, as described (13). Human solid tumor-based cell lines obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 with 5% FBS. Cell Growth Assay. Adherent human solid tumor cells plated in 96-well plates were treated for 5 days in the presence and absence of various doses of EPA ranging from 20 to 100 μM in RPMI 1640 containing 5% FBS. At the time of termination, cells were stained with sulforhodamine B and counted as described (27).

Mitogenic Assay. Synthesis of DNA by incorporation of [3H]thymidine was determined as described (12). Briefly, 4 × 104 NIH 3T3 cells, cells transfected with either vector, PKR-K296, or eIF2α-51A, were made quiescent by serum withdrawal for 36 h (0.1% calf serum/DMEM) and then exposed to a mitogenic stimulus (5 ng/ml bFGF) in the absence or presence of different concentrations of EPA. After 15 h, 1 μCi/ml [3H]thymidine (DuPont NEN, Boston, MA) was added, and the cells were incubated at 37°C in 5% CO2 for 6 h. Cells were harvested and counted in a Packard Top Count (Packard, CT).

Ca2+ Assays. Exponentially growing cells were harvested, washed with PBS, and resuspended in Krebs-Ringer medium buffered with 25 mM Hepes (pH 7.4 at 37°C). Cells were loaded in the same buffer with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR), and 4 × 106 were used for cytosolic Ca2+ measurement with a Photon Technology International dual excitation spectrophotometer (South Brunswick, NJ), exactly as described (28).

Polysome Profile Analysis. Exponentially growing NIH 3T3 cells were exposed to EPA (30 μM) for 2 h in DMEM/5 mg/ml bFGF/0.1% calf serum, followed by addition of cycloheximide (25 μg/ml), and polysome profiles were determined exactly as described (13, 29).

Protein Synthesis. Total protein synthesis was measured as described (13). Briefly, NIH 3T3 cells and stable transfected cells were treated with EPA for 1 h and pulse labeled with [35S]Met-Cys for 15 min, and incorporation of label into TCA-precipitated total protein determined.

Western and Northern Blot Analyses. Protein extraction and Western blot analysis were done as described (30). Total protein extract (40 μg/lane) was analyzed on blots incubated overnight in specific antibodies (Santa Cruz Laboratories, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were detected by ECL kit (Pierce, Rockford, IL). RNA (25 μg) was fractionated on a 1% formaldehyde agarose gel, blotted onto nylon membranes, and hybridized with either cyclin D1 or 18S RNA cDNA probe labeled by random-prime labeling with [α-32P]dCTP as described (13).

Phosphorylation of eIF2α. Exponentially growing NIH 3T3, PKR-K296, eIF2α-51A, or vector control stable transfected cells were treated with 32P-orthophosphoric acid (200 μCi/ml) for 3.5 h. One-half of the cells were challenged with EPA (30 μM) for 0.5 h, lysed with IP buffer, and RNase-DNase treated. TCA precipitable counts were determined, and equal number of counts were immuno-precipitated with anti-eIF2α antibody (kindly provided by Dr. N. Sonenberg, McGill University, Montreal, Quebec, Canada) and separated by SDS-PAGE; phosphorylation of eIF2α was quantified by PhosphorImager as described (13).

Cell Cycle Analysis. NIH 3T3 cells (1 × 107) were treated with 7.5 μM EPA for up to 48 h in DMEM with 2% calf serum and 5 ng/ml bFGF, changed each day. The cells were fixed with ethanol and stained with propidium iodide for cell cycle analysis by flow cytometry.

Animal Studies. To establish the KLN-205 squamous cell carcinoma model, KLN-205 tumor cells were grown in culture, harvested and injected as a suspension (2.5 × 106 cells in 0.1 ml of PBS) s.c. into 6-week-old female DBA/2J mice (Jackson Laboratories). Four days after tumor implantation, mice received either vehicle or EPA (2.5 g EPA/kg body weight) in the form of a lipid concentrate by gavage daily for 5 weeks. Three-dimensional tumor size was measured by calipers every week, and volumes were calculated using the equation: v (in mm3) = width × length × height. Statistical differences between tumor volumes in control and the EPA-treated group was determined by Student’s t test. The general health of the animals was monitored during the treatment period. At the end of the study, animals were sacrificed by CO2 asphyxiation, and tumors were excised and fixed in 10% buffered formalin.

Expression of Cyclin D1 in Tumor Tissue. To analyze for cyclin D1 expression, 4-μm sections of formalin-fixed, paraffin-embedded tumor tissues from all of the animals in each group were cut and fixed on slides. Sections were deparaffinized in graded alcohol, immunostained with anti-cyclin D1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and counterstained with hematoxylin. A minimum of three different areas from each section per animal were analyzed for cyclin D1 using BioQuant software. The percent labeling index for cyclin D1 was calculated by the formula: (cyclin D1 positive nuclei/total nuclei) × 100.

DNA Analysis for Ploidy Levels and Tumor Cell Kinetics. Formalin-fixed, paraffin-embedded tumor sections were stained by the Feulgen method and microscopically analyzed for DNA content using the BioQuant system. The DNA content was measured in 350 tumor cell nuclei selected randomly from distinct areas of all slides from each tissue group. The proportion of cell populations with differing DNA content were estimated by peak fitting using Jandel software that identifies normally distributed populations in the data. The area percent under the fitted curves represents cells with differing ploidy content. A Feulgen-stained mouse liver sample was used to verify the 2C DNA content. A 2C content characterizes cells in G0-G1, a 2C-4C content characterizes cells in S phase, and a 4C content, cells in G2-M phase. Malignant tumors characteristically show a cell population containing hypertetraploid (>4C) DNA content, considered an index of tumor malignancy (31, 32).

RESULTS AND DISCUSSION

EPA Inhibits the Growth of Cancer Cells and DNA Synthesis in NIH 3T3 Cells in Vitro. The effect of EPA on cell growth was assessed in a diverse panel of human cancer cells using the sulforhodamine B assay. Exposure of cancer cells to EPA for 5 days in a medium containing 5% FCS inhibited cell growth with a half maximal inhibition in the range of 20–100 μM (Table 1). In quiescent NIH 3T3 cells stimulated in DMEM/bFGF (5 ng/ml)/0.1% calf serum, EPA inhibited DNA synthesis, as measured by [3H]thymidine incorporation, with half-maximal inhibition at 7.5 μM (Fig. 1).

EPA Releases Intracellular Ca2+ and Blocks Capacitative Ca2+ Influx. There is an established relationship between the filling state of the intracellular Ca2+ pools and cell proliferation (33–35). Indeed, we have demonstrated that compounds such as clotrimazole inhibit proliferation of normal and cancer cells in vitro and tumor growth in vivo because they induce sustained partial depletion of intracellular Ca2+ stores (12). Because EPA releases Ca2+ from internal stores (25) and at the same time inhibits their refilling via capacitative Ca2+ influx (26), we predicted that EPA would likely induce partial Ca2+ store depletion. This prediction was confirmed by the finding that in

<table>
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<th>Cancer cells</th>
<th>Type</th>
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<tbody>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>23.5 ± 1.9</td>
</tr>
<tr>
<td>Hep-G2</td>
<td>Liver</td>
<td>24.1 ± 1.1</td>
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<tr>
<td>U-118 MG</td>
<td>Glioblastoma</td>
<td>36.0 ± 3.2</td>
</tr>
<tr>
<td>DU-145</td>
<td>Prostate</td>
<td>36.4 ± 2.3</td>
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<td>MDAMB231</td>
<td>Breast</td>
<td>37.7 ± 2.8</td>
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<tr>
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<td>Breast</td>
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<tr>
<td>SK-OV3</td>
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EPA Inhibits Protein Synthesis at the Level of Translation Initiation.

The filling state of the Ca^{2+} pools influences the rate of cellular protein synthesis (38). To investigate whether EPA inhibits protein synthesis, NIH 3T3 cells were treated with EPA and pulse labeled with [35S]Met-Cys, and we determined the incorporation of label into total protein. EPA inhibited protein synthesis (Fig. 3a) in a dose-dependent manner (Fig. 3a, inset). The rate of total protein synthesis as a function of EPA concentration reaches a minimum plateau at (~15 μM), a value comparable with the concentration required for half-maximal inhibition of protein synthesis in ~33% of control (Fig. 3a); this is most likely because EPA only inhibits synthesis of a subset of proteins whereas other proteins are not affected, as we will show below. Fig. 3 shows that the concentration of EPA required for half-maximal inhibition of protein synthesis is ~15 μM, a value comparable with the concentration required for half-maximal inhibition of cell growth (Fig. 1). Similar protein synthesis inhibition was obtained with our cancer cell panel (data not shown). To investigate whether EPA inhibits protein synthesis as a direct result of Ca^{2+} store depletion, exponentially growing NIH 3T3 cells were transiently incubated with or without EPA for 1 h. The cells were washed with Ca^{2+}-free medium to remove EPA and also to render the media Ca^{2+} free. Cells were then pulse-labeled with [35S]Met-Cys in the presence or absence of external Ca^{2+}, and incorporation of label was determined as above. Cells transiently exposed to EPA reinitiated protein synthesis only when external Ca^{2+} was present. EPA also reverses the antiproliferative activity of EPA, suggesting that the anticancer effect of EPA is mediated through lipid peroxidation (36, 37). To evaluate whether peroxidation products of EPA are responsible for EPA-induced Ca^{2+} release, experiments were performed in NIH 3T3 cells cultured in the presence of vitamin E (100 μM) prior to their loading with Fura-2 AM and exposure to EPA. EPA did not cause Ca^{2+} release in cells cultured with vitamin E (Fig. 2h, lower curve). In contrast, pretreatment with vitamin E did not affect the Ca^{2+} release induced by clotrimazole or thapsigargin (data not shown) indicating that vitamin E does not affect the ability of the cellular Ca^{2+} stores to release Ca^{2+}, and that peroxidation products of EPA induce Ca^{2+} release. Together, these results provide a direct evidence for a link between the effect of EPA on Ca^{2+} pools and its growth-inhibitory activity and indicate that these effects are related to a peroxidation product(s) of EPA on the filling state of the cellular Ca^{2+} pools.

EPA Inhibits DNA Synthesis in NIH 3T3 Cells. DNA synthesis was measured by [3H]thymidine incorporation in confluent, quiescent NIH 3T3 cells stimulated with DMEM/0.1% calf serum/bFGF (5 ng/ml) in the presence and absence of EPA. Bars, SE.

Fig. 1. EPA inhibits DNA synthesis in NIH 3T3 cells. DNA synthesis was measured by [3H]thymidine incorporation in confluent, quiescent NIH 3T3 cells stimulated with DMEM/0.1% calf serum/bFGF (5 ng/ml) in the presence and absence of EPA. Bars, SE.

Fig. 2. EPA releases intracellular Ca^{2+}, inhibits capacitative Ca^{2+} influx, and abrogates mitogen-induced Ca^{2+} transients. Fura-2 AM-loaded NIH 3T3 (a, c) or cancer (d) cells were suspended in Ca^{2+}-free medium or in medium containing 2 mM CaCl_2 (c), and the cytosolic Ca^{2+} was monitored continuously. Calcium release was stimulated with 500 ng/ml bFGF after 5 min preincubation with or without 30 μM EPA (a), with 30 μM EPA (b and d), or with 300 nM thapsigargin (c). For the experiment shown in b, cells were incubated with or without 100 μM vitamin E for 24 h before testing the effect of EPA. Note that in a, the concentration of bFGF required to elicit cytosolic Ca^{2+} changes is 100-fold higher than that used for the mitogenic assay in Fig. 1 because 100 times more cells were used for the Ca^{2+} measurements. d, inset, Ca^{2+} release after 120 μM EPA addition to HTB-174 cells.
supplied; pulse labeling in the absence of external \( \text{Ca}^{2+} \) resulted in marked inhibition of protein synthesis. In contrast, removal of external \( \text{Ca}^{2+} \) during the pulse labeling period in control cells not exposed to EPA had negligible effect on protein synthesis. This experiment indicates that inhibition of protein synthesis by EPA is the direct result of \( \text{Ca}^{2+} \) store depletion (Fig. 3b).

Depletion of intracellular \( \text{Ca}^{2+} \) stores activates PKR that in turn phosphorylates eIF2\(\alpha\) and inhibits translation initiation, the rate-limiting step of protein synthesis (39–41). To investigate whether EPA inhibits protein synthesis at the level of translation initiation, we determined the polyribosome profiles in sucrose density fractions derived from exponentially growing NIH 3T3 cells incubated with EPA for 2 h. EPA shifted the ribosomal profile from heavy polyribosomes to lighter polysomes, monosomes, and free ribosomal subunits (Fig. 4). This shift toward lighter fractions clearly demonstrates that EPA inhibits translation initiation.

Translation in eukaryotic cells starts with the assembly of the eIF2-GTP-Met-tRNA ternary complex, recruitment of the 40S ribosomal subunit, followed by binding to mRNA at its 5’end. The ribosomal complex then migrates along the 5’ UTR of the mRNA in a process facilitated by an array of initiation factors including eIF2 and eIF4 (42). At the end of each initiation event, GTP is hydrolyzed, and the eIF2-GDP complex is released. Regeneration of the eIF2-GTP complex by a GDP-GTP exchange reaction catalyzed by eIF2B is required to start a new round of translation initiation. Phosphorylation of serine 51 in the \( \alpha \) subunit of eIF2 by PKR inhibits GDP-GTP exchange, suppressing the initiation of translation. To investigate whether the inhibitory effect of EPA on translation initiation is attributable to phosphorylation of eIF2\(\alpha\) by PKR, cells expressing either a nonphosphorylatable mutant of eIF2\(\alpha\) (eIF2\(\alpha\)-51A; Ref. 19) or a dominant-negative mutant of PKR [PKR-K296; which has no enzymatic activity and acts as a dominant-negative mutant because of its ability to heterodimerize with endogenous protein (43)] were tested for their sensitivity to EPA. Compared with vector-transfected cells, eIF2\(\alpha\)-51A and PKR-K296 transfected cells were resistant to the inhibitory action of EPA on both cell growth and protein synthesis (Fig. 5, a and b). This observation does not reflect a general resistance of the eIF2\(\alpha\)-51A and PKR-K296 transfected cell lines to antiproliferative agents because their sensitivity to etoposide was similar to that of vector-transfected control cells (data not shown). These results indicate that PKR-mediated phosphorylation of eIF2\(\alpha\) is responsible for the inhibitory effect of EPA on protein synthesis and cell growth.

To confirm this interpretation, we measured phosphorylation of eIF2\(\alpha\) in response to EPA treatment. Exponentially growing NIH 3T3 cells transfected with vector only, eIF2\(\alpha\)-51A, or PKR-K296 were incubated with \( ^{32} \text{P} \text{Pi} \) to label the intracellular ATP pool and challenged with EPA. Phosphorylation of eIF2\(\alpha\) was determined by the amount of \( ^{32} \text{P} \) incorporated into immunoprecipitated eIF2\(\alpha\). EPA treatment markedly increased eIF2\(\alpha\) phosphorylation in both parental NIH 3T3 (not shown) and vector-transfected cells; in contrast, phosphorylation of eIF2\(\alpha\) was minimal in eIF2\(\alpha\)-51A and PKR-K296 transfected cell lines (Fig. 5c). It has been reported recently that mouse embryonic fibroblasts that are nullizygous for PKR are not impaired in their ability to inhibit protein synthesis in response to endoplasmic reticulum stress (44). This is because the endoplasmic reticular-resident kinase PERK that is also activated by \( \text{Ca}^{2+} \) depletion phosphorylates eIF2\(\alpha\) on serine 51 and thereby inhibits mRNA translation. Our results showing abrogation of eIF2\(\alpha\) phosphorylation in PKR-K296 transfected cells treated with EPA indicate that PERK is not involved in the inhibition of protein synthesis that we observe in response to EPA. Alternatively, PKR-K296 may also block PERK activity and/or compete for a rate-limiting cofactor required for phosphorylation of eIF2\(\alpha\).

EPA Preferentially Abrogates G1 Cyclin Synthesis and Expression and Causes Cell Cycle Arrest in G1. Reducing the rate of translation initiation preferentially inhibits the synthesis of proteins that are inefficiently translated (45, 46), mostly because they are coded for by mRNAs with highly structured 5’ UTRs. Because the mRNAs of G1 cyclins contain highly structured 5’ UTRs, their synthesis and expression requires optimal activity of translation initiation factors (16). Thus, pharmacological agents that decrease the rate of translation initiation are expected to inhibit preferentially synthesis and expression of G1 cyclins and other cell growth-regulatory proteins (15). Exponentially growing NIH 3T3 cells were treated with EPA and \( ^{35} \text{S} \text{Met-Cys} \) for 1 h and assayed for incorporation of \( ^{35} \text{S} \text{Met-Cys} \) into cyclin D1, cyclin E, and Ras, as well as into \( \beta \)-actin and ubiquitin. EPA inhibited synthesis of cyclin D1, cyclin E, and Ras, all coded for by mRNAs with highly structured 5’ UTRs, but not of \( \beta \)-actin or ubiquitin, proteins coded for by mRNAs with simple 5’ UTRs (Fig. 6a). EPA treatment also resulted in a parallel reduction in the expression of cyclin D1 and cyclin E, whereas expression of \( \beta \)-actin and ubiquitin was not affected (Fig. 6a).

Because cyclin D1 has a short half-life of ~30 min (47), the effect of EPA on incorporation of \( ^{35} \text{S} \text{Met-Cys} \) into cyclin D1 and on cyclin D1 expression could be attributable to reduced synthesis or increased degradation.
degradation. In contrast, the effect of EPA on Ras is unlikely attributable to an increased degradation of this protein because it has a longer half-life that is comparable with that of ubiquitin or β-actin (>16 h). These results support the conclusion that EPA exerts a differential inhibitory effect on the synthesis and expression of a subset of proteins including G1 cyclins. This is further confirmed by the finding that in PKR-K296 and eIF2α-51A transfected cells that are resistant to its growth-inhibitory effect, EPA did not affect cyclin D1 or cyclin E expression (Fig. 6c). EPA-induced inhibition of translation initiation may explain the reduced expression of farnesyl protein transferase, an enzyme essential for the transforming activity of ras oncoproteins in EPA-treated mice with colon cancer (48).

To determine whether the effect of EPA on synthesis and expression of cyclin D1 is primarily mediated through inhibition of trans-
lation initiation rather than transcription, quiescent NIH 3T3 cells were stimulated with bFGF for 8 h and then treated with or without EPA for an additional hour. Cells were tested simultaneously for the synthesis of cyclin D1 protein and expression of cyclin D1 mRNA. EPA treatment strongly inhibited cyclin D1 synthesis without affecting the expression of its mRNA (Fig. 6b). Furthermore, Northern blot analysis with a cyclin D1-specific probe of mRNAs isolated from polysome fractions (described in Fig. 4) showed a significant shift of cyclin D1 mRNA from heavier to lighter polysomal fractions (Fig. 6d). These experiments confirm that EPA inhibits cyclin D1 synthesis at the level of translation initiation. Importantly, they also demonstrate that EPA does not affect cyclin D1 transcription, indicating that EPA inhibits cell growth without interfering with growth factor signaling upstream from cyclin D1 transcription.

In eukaryotic cells, successive expression of cyclin D1 and E during the G1 phase of the cell cycle is critical for progression beyond the restriction point and into S phase. To test whether EPA blocks cell cycle progression before the restriction point, quiescent NIH 3T3 cells were stimulated with bFGF, and the effect of adding EPA at hourly intervals on entry into S phase was monitored. Quiescent cells enter S phase 12–13 h after bFGF stimulation (13). EPA addition to these cells until late G1 (10–11 h after bFGF stimulation) prevented G1-S progression, even when cells were exposed to EPA for only 1 h before beginning of S phase. In contrast, the addition of EPA at later times failed to inhibit DNA synthesis (data not shown), indicating that EPA blocks cell proliferation specifically in G1, most probably before the restriction point. These results were further confirmed by FACS cell cycle analysis of propidium iodide-stained cells. Treatment with EPA (7.5 μM) for 48 h resulted in a significant accumulation of cells in G1: Control cells: G0-G1, 65%; S, 31%; G2-M, 4%; EPA-treated cells: G0-G1, 87%; S, 11%; G2-M, 2%. These results indicate that by reducing synthesis and expression of G1 cyclins, EPA blocks cell cycle progression in G1.

**EPA Reduces Tumor Growth and Induces G_0-G_1 Arrest in Vivo.** The in vivo consequences of EPA-mediated inhibition of translation initiation were elucidated in the KLN mouse squamous cell carcinoma model. Starting 4 days after inoculation, either vehicle or marine lipid concentrate containing EPA was administered daily by gavage at a dose of 2.5 g/kg for 5 weeks (n = 10 animals/group). Tumors were measured on a weekly basis for 5 weeks and then analyzed by immunohistochemistry for expression of cyclin D1 and by Feulgen stain for nuclear DNA content. EPA treatment significantly reduced tumor growth (Fig. 7a) while showing no signs of toxicity, as indicated by the absence of weight loss or any behavioral abnormalities with treatment. Consistent with the effect of EPA on G1 cyclin expression in cultured cells, EPA-treated tumors showed a significant decrease in the number of cells expressing cyclin D1 (Fig. 7b).
EPA reduced the cyclin D1 labeling index from 29 ± 5 in vehicle-treated animals to 4 ± 1 (mean ± SE, P < 0.001). Nuclear DNA content analysis of tumors documented that EPA significantly increased the number of cells with a diploid (2C) DNA content, indicating cell cycle arrest in G$_0$/G$_1$. In addition, it significantly decreased the number of hypertetraploid cells (>4C DNA content), representative of the rapidly dividing malignant cell population (Fig. 8).

The results reported in this paper demonstrate that EPA and/or its peroxidation product(s) inhibits cell proliferation by causing partial but sustained depletion of intracellular Ca$^{2+}$ stores. As a result of Ca$^{2+}$ store depletion, EPA causes cell cycle arrest in G$_2$ because it inhibits translation initiation and thereby preferentially abrogates the synthesis and expression of G1 cyclins both in vitro and in vivo. This mechanism of the antiproliferative action of EPA closely resembles that of chlorotiazole, another translation initiation inhibitor that shares with EPA potent antiproliferative effects in tumor cells in vitro and in tumor-bearing animals with very low toxicity, even after prolonged daily administration. Furthermore, the inhibitory effect of EPA on translation initiation and its preferential inhibitory action on the synthesis of growth-regulatory proteins provides a possible explanation for the epidemiological evidence that populations consuming food rich in EPA and other n-3 PUFAs have a low incidence of cancer.

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Inhibition of Translation Initiation Mediates the Anticancer Effect of the n-3 Polyunsaturated Fatty Acid Eicosapentaenoic Acid

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