Growth Factor Modulated Calmodulin-Binding Protein Stimulates Nuclear DNA Synthesis in Hemopoietic Progenitor Cells†

G. Prem Veer Reddy,*,‡,§ William C. Reed,‖ Donna H. Deacon,⊥ and Peter J. Quesenberry‡

Cancer Center and Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605, and Department of Obstetrics and Gynecology and Department of Hematology Oncology, University of Virginia, Charlottesville, Virginia 22906

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ABSTRACT: A specific calmodulin-binding protein of 68 kDa (CaM-BP68) is modulated in response to growth factors that induce proliferative stimulation in a variety of hemopoietic progenitor cells. The nuclear localization of the CaM-BP68 coincided temporally with interleukin 3 (IL-3)-dependent progression of synchronized FDC-P1 cells from G1 to S phase [Reddy et al. (1992) *Blood 79*, 1946–1956]. To delineate the role of the CaM-BP68 in the onset of DNA synthesis (S phase), this protein was purified to an apparent homogeneity from FDC-P1 cells and its effects on DNA replication in permeabilized FDC-P1 cells were examined. Purified CaM-BP exhibited a single silver-stained protein band of 68 kDa on SDS–polyacrylamide gels. This purified protein, when incubated with permeabilized log-growing FDC-P1 cells, caused a 3–4-fold increase in the rate of [^{3}H]dTTP incorporation into DNA as compared to the controls. There was a direct correlation between the increase in the rate of [^{3}H]dTTP incorporation into DNA and the concentration of the added CaM-BP68 in the incubation mixture. These observations suggest that the CaM-BP68, whose nuclear localization is associated with growth factor dependent proliferative stimulation of myeloid progenitor cells, is involved in the regulation of nuclear DNA synthesis.

Progression of mammalian cells through G1 and into S phase is regulated by a series of events that are determined by extracellular signals generated by growth factors (Pardee, 1989; Moses & Pilistine, 1985; Hill & Milner, 1985; Straus, 1984; Rothstein, 1982). While the temporal order of the growth factor requirement for the progression of cells from G1 to S phase has been studied extensively (Pledger et al., 1977, 1978; Campisi & Pardee, 1984), the molecular mechanism by which growth factors induce the onset of DNA synthesis (S phase) remains unknown.

Interactions between extracellular growth factors and their cognate receptors on cell membrane are often associated with the stimulation of membrane-bound phospholipase C. This catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3). DG is known to activate and redistribute protein kinase C (Wolfman & Macara, 1987), which in turn phosphorylates serine and/or threonine residues of its target proteins, including adenylate cyclase (Sibley et al., 1988). IP_3 causes the release of Ca^{2+} from intracellular stores (Hokin, 1985). The role of activated protein kinase C and cyclic nucleotides in cellular proliferation has been the subject of conflicting observations (Whitfield et al., 1987; Persons et al., 1988; Besterman et al., 1986; Jacob & Cautrecasas, 1986). The involvement of Ca^{2+} and its receptor protein, calmodulin (CaM),[1] in the regulation of mammalian cell proliferation, particularly at the G1/S boundary, has been demonstrated through biochemical, pharmacological, and genetic studies in a variety of cell systems (Lu & Means, 1993; Means & Rasmussen, 1988; Whitfield et al., 1988). Recently, it has been established that DNA replication in S-phase fibroblasts is highly sensitive to CaM-specific monoclonal antibodies (Reddy et al., 1992b). Consistent with the involvement of CaM in growth factor dependent progression of mammalian cells from G1 to S and in the process of DNA synthesis are the observations that the expression and the nuclear localization of a specific calmodulin-binding protein (CaM-BP) of 68 kDa is associated with the growth factor-dependent progression of mammalian cells from G1 to S phase (Subramanyam et al., 1990; Reddy et al., 1992a). This protein is modulated in response to insulin or IGF-1 as the synchronized fibroblasts transit from G1 to S phase and is associated with the replitase complex responsible for nuclear DNA replication in S-phase cells (Subramanyam et al., 1990). Furthermore, a similar CaM-BP68 also exhibits a stringent cytokine-dependent modulation in a variety of factor-dependent hemopoietic progenitor cells (Reddy et al., 1992a). The nuclear localization of CaM-BP68 coincided temporally with IL-3-dependent progression of synchronized FDC-P1 cells from G1 to S phase (Reddy et al., 1992a). These observation suggest that the growth factor-induced proliferation of a variety of mammalian cells may be associated with a common mechanism involving nuclear localization of a CaM-BP68, but there is no direct evidence for a role for this protein in cell cycle progression from G1 to S phase or in DNA synthesis.

To discern the mechanism of CaM action in cell proliferation, and to identify the biological significance of the growth factor-dependent modulation of myeloid CaM-BP68, we have purified the CaM-BP68 from the nuclear lysate of FDC-P1

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* Address correspondence to this author at Cancer Center, University of Massachusetts Medical Center, 373 Plantation St., Suite 202, Worcester, MA 01605 [telephone (508) 856-6956; fax (508) 856-1310].

‡ Cancer Center.

§ Department of Cell Biology.

‖ Department of Obstetrics and Gynecology.

⊥ Department of Hematology Oncology.

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[1] Abbreviations: CaM, calmodulin, CaM-BP, calmodulin-binding protein; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium lauryl sulfate; $AmSO_4$, ammonium sulfate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; BSA, bovine serum albumin.

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cells and examined its effects on nuclear DNA replication of the same cells. Our observation that the purified CaM-BP68 causes a 3–4-fold increase in DNA synthesis of permeabilized FDC-P1 cells provides direct evidence for the involvement of a specific calmodulin-binding protein, and therefore of CaM, in cellular proliferation.

MATERIALS AND METHODS

Cell Lines and Their Cultures. FDC-P1 cells (obtained from Dr. Jim Ihle, St. Jude's Hospital, Memphis, TN) were maintained routinely in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 25% WEHI-3 cell conditioned medium (WEHI-3 cm). M07e cells (obtained from Genetics Institute, Cambridge, MA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% heat-inactivated FCS and 100 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). Both of the cell culture media contained 100 U/mL penicillin and 100 μg/mL streptomycin, and the cultures were maintained in an incubator containing 10% CO_2. Deprivation of growth factors and the subsequent readdition of interleukin 3 (IL-3) (200 U/mL, for FDC-P1 cells) or of GM-CSF (100 U/mL, for M07e cells) was performed as described elsewhere (Reddy et al., 1992a).

Preparation of Nuclear Lysate. The nuclear lysate for the detection of calmodulin-binding proteins (CaM-BPs) in FDC-P1 and M07e cells was prepared following homogenization of the cells in buffer A [35 mM Hepes, pH 7.4, 150 mM sucrose, 80 mM KCl, 5 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 8 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride (PMSF)] at a density of 5×10^7 cells/mL as described (Reddy et al., 1992a).

Identification of CaM-BPs. Protein samples (nuclear lysate and various fractions collected through the multiple steps of the CaM-BP68 purification described below) were subjected to SDS–polyacrylamide gel electrophoresis using a mini-gel apparatus (Bio-Rad, Richmond, CA). Proteins resolved on 10% polyacrylamide gels were transferred to nitrocellulose filters at 200 mA for 1–2 h using a Hoefer Scientific Transphor Electrophoresis unit. CaM-BPs on the filters were then identified by using biotinylated CaM (purchased from Biomedical Technologies, Inc., Stoughton, MA) and alkaline phosphatase-conjugated avidin (purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN) as described by Billingsley et al. (1985). Prestained molecular weight markers (purchased from Bethesda Research Laboratories, Bethesda, MD) were used to identify the relative molecular weight of CaM-BPs detected on the filters. Filters were air-dried and photographed.

Purification of the CaM-BP68 from Nuclear Lysate. FDC-P1 cells grown to a density of $1–1.5 \times 10^6$ cells/mL in spinner cultures were harvested and the nuclear lysate was prepared in buffer A as described by Reddy et al. (1992a). The nuclear lysate cleared by centrifugation at 10000*g* and 4 °C for 10 min was treated with 0.1 g/mL ammonium sulfate at 4 °C for 30 min and centrifuged again. The supernatant was once again treated with 0.1 g/mL ammonium sulfate and centrifuged. The pellet was then dissolved in buffer A at one-fifth the volume of the nuclear lysate. After centrifugation at 5000*g*, the supernatant contained approximately 80% of the nuclear CaM-BP68. This supernatant was applied to a Sephacryl S-200 gel (Sigma Chemical Co., St. Louis, MO) filtration column with a bed volume of 3 × 75 cm, the column was developed at 1 mL/min with buffer B (20 mM Tris, pH 7.4, 4 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM KCl, and 1 mM PMSF), and 7-mL fractions were collected. Active fractions containing CaM-BP68 were pooled and subjected to anion-exchange high-performance liquid chromatography (HPLC) with a DEAE-MemSep 1010 cartridge (Millipore Corp., Beford, MA) on a Gilson System 42 (Gilson Medical Electronics, Inc., Middleton, WI), pre-equilibrated with buffer B. Thirty to 40 mL of the pooled fractions from the Sephacryl S-200 column was filtered sequentially through 0.8-, 0.45-, and 0.2-μm low-protein binding Acrodisc syringe filters (Gelman Sciences, Ann Arbor, MI) and then applied to the DEAE cartridge at 21 °C and a flow rate of 2 mL/min. The cartridge was then washed with buffer B at a flow rate of 5 mL/min until the absorbance of the eluting buffer at 250 nm reached baseline. Bound proteins were eluted with a linear gradient of 0.01–0.5 M KCl in buffer B over 20 min. Fractions of 10 mL were collected and assayed for CaM-BPs. The CaM-BP68 containing fractions were pooled and dialyzed against a 25-fold excess buffer B for 6 h at 4 °C. A column containing calmodulin–agarose (purchased from Sigma) (1 × 7 cm) was equilibrated with buffer B. Dialyzed CaM-BP68 containing fraction was then applied to the CaM–agarose column at a flow rate of 0.75 mL/min and washed with buffer B until the absorbance of the eluting buffer stabilized at the baseline. Elution was performed with buffer B containing 10 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) instead of 2 mM $CaCl_2$. CaM-BP assays were performed on each 5-mL fraction, and the active fractions were pooled and separated on a AXXI-CHROM analytical C_8 octyl 5-μm HPLC column (0.46 × 15 cm) (purchased from Cole Scientific Inc., Moorpark, CA) at 21 °C. The column was equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) (Sigma) in water] at a flow rate of 0.8 mL/min and the sample applied to the column. After washing, the column was eluted with a linear gradient of solvent B (0.09% TFA in acetonitrile) from 20% to 80% over 10 min. Fractions containing the CaM-BP68 were dialyzed against buffer B, adjusted to 10% glycerol, and stored at −20 °C. CaM-binding activity of the 68-kDa protein is stable for several weeks under these conditions.

Protein Estimation. Protein content in individual fractions was determined according to the method of Lowry et al. (1951).

Permeabilization of FDC-P1 Cells. Fewer than 20% of the cells in FDC-P1 cultures grown to a density of about 1.5×10^6 cells/mL were actually in S phase (data not shown). Cells from such cultures were harvested, washed once with buffer A, and permeabilized by treating with 0.5 mg/mL L-α-lysophosphatidylcholine (lysolecithin) as described by Reddy and Pardee (1982). These permeabilized cells were then employed for testing the effect of purified CaM-BP68 on the incorporation of exogenous deoxynucleotides into their DNA.

Measurement of Rate of DNA Synthesis in Permeabilized FDC-P1 Cells. Incorporation of [^{3}H]dTTP into DNA of permeabilized cells was monitored by incubating 32 μL of permeabilized cells (1×10^8 cells/mL) with the DNA replication reaction mixture containing 35 mM Hepes (pH 7.4), 150 mM sucrose, 80 mM KCl, 4 mM $MgCl_2$, 7.5 mM potassium phosphate (pH 7.4), 0.075 mM $CaCl_2$, 10 mM phospho(enol) pyruvate, 2 mM rATP, 30 μM each of rGTP and rCTP, 100 μM each of dATP, dGTP, and dCTP, 50 μM [^{3}H]dTTP (70 cpm/pmol), and 12 μL of 0.4 mg/mL BSA or purified CaM-BP68 (0.4 mg/mL), in a final volume of 0.075 mL. Incubations were performed at 37 °C, and the radioactivity incorporated into 30-μL duplicate aliquots of each incubation was determined as described elsewhere (Reddy

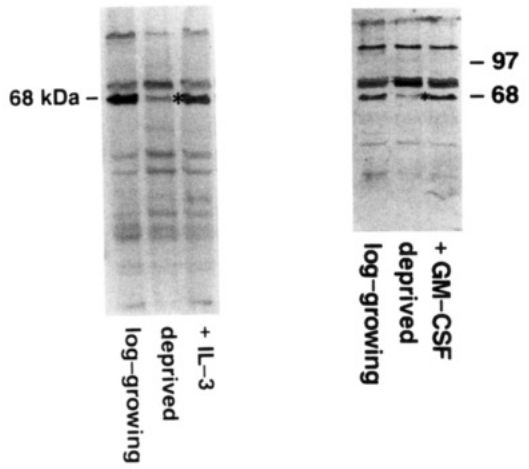


FIGURE 1: Effect of IL-3 and GM-CSF on restoration of nuclear CaM-BP68 in cytokine-deprived FDC-P1 (A, left) and MO7e (B, right) cells. Log-growing FDC-P1 and MO7e cells were deprived of cytokines for 20 h (deprived), and 6 h following addition of IL-3 (+IL-3) or GM-CSF (+GM-CSF) nuclear lysates were prepared and CaM-BPs determined as described under Materials and Methods.

et al., 1992b; Reddy & Pardee, 1982). These reaction conditions and the assay procedures were identified to be optimal for the measurement of DNA replication in permeabilized fibroblast cells (Reddy et al., 1992b; Reddy & Pardee, 1982).

RESULTS

Myeloid progenitor FDC-P1 cells have been the primary choice in these studies because of their requirement for IL-3 or GM-CSF for growth and nuclear localization of the CaM-BP68. When the medium was supplemented with 10% fetal calf serum, but no cytokines, cells failed to proliferate and the CaM-BP68 was markedly decreased in the nuclear fraction (Figure 1A). Addition of IL-3 alone restored nuclear CaM-BP to the levels observed in log-growing cultures (Figure 1A) and enabled the cells to proliferate. This observation is not unique to the rodent cells, since factor-dependent human M07e cells also exhibit stringent requirement for GM-CSF for their proliferation and for the nuclear localization of the CaM-BP68 (Figure 1B). Thus, there is evidence for the regulation of a CaM-BP68 in association with proliferative stimulation in both rodent and human cells in reponse to growth factors.

Purification and Properties of the Nuclear CaM-BP68 from FDC-P1 Cells. Log-growing FDC-P1 cells were used for the purification of the nuclear CaM-BP68. This protein could not be purified from cultures that were either deprived of the cytokines or grown to a cell density greater than 2–3 × 10^6 cells/mL (data not shown). Such cells contained dramatically reduced levels of CaM-BP68 (Figure 1). During the course of development of the overall purification scheme for the CaM-BP68, it was observed that the CaM-binding property of this protein was optimal at pH 6.5–8 and remained stable for several days when stored at 4 °C but decreased dramatically within 5 min when the temperature was increased above 50 °C.

Nuclear lysate prepared from log-growing FDC-P1 cells routinely contains approximately 50% of the total cellular CaM-BP68 (Figure 2). Addition to the nuclear lysate of ammonium sulfate to a concentration of 35% precipitates the majority of the CaM-BP68 (Figure 2) by excluding about 75% of other nuclear proteins from this fraction (Table 1). As shown in Figure 3, Sephacryl S-200 gel filtration of the dissolved 35% ammonium sulfate precipitate separates

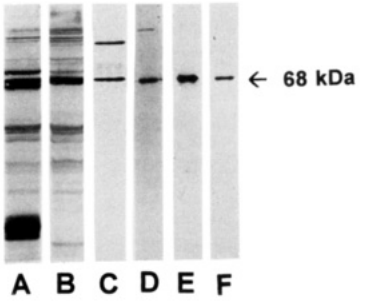


FIGURE 2: Profile of calmodulin-binding proteins in active fractions at various steps of CaM-BP68 purification. (Lane A) Nuclear lysate; (lane B) ammonium sulfate (35%) precipitate of nuclear lysate; (lane C) Sephacryl S-200 gel filtration of 35% ammonium sulfate precipitate; (lane D) anion-exchange HPLC of active fractions from Sephacryl S-200 gel filtration; (lane E) CaM–agarose affinity chromatography of active fractions from anion-exchange HPLC; (lane F) reversed-phase HPLC of active fractions from CaM–agarose affinity chromatography. Experimental procedures for each of these purification steps and for the determination of calmodulin-binding proteins in individual fractions were as described under Mateials and Methods.

Table 1: Purification of the Calmodulin-Binding Protein

purification step	protein concn (mg/mL)	total protein (mg)	protein enrichment[a] (-fold)
(1) whole cell extract (2.5 × 10^9 cells)	6.8	341	1
(2) nuclear fraction	4.7	165	2
(3) 35% $AmSO_4$ precipitation	3.4	34	10
(4) S-200 gel filtration	0.23	10.6	32
(5) anion-exchange HPLC	0.076	3.1	110
(6) CaM–agarose affinity chromatography	0.112	1.23	277
(7) C_8 reversed-phase HPLC	0.035	0.141	2418

[a] Protein enrichment was estimated from the decrease in total protein while the relative detectability of the CaM-BP68 in purified fractions remained approximately the same as compared to the whole cell extract.

the CaM-BP68 with fractions 15–17 that are eluted at a molecular weight higher than the expected 68 000 determined by SDS–PAGE analysis. A similar observation is made even when the nuclear lysate is applied directly (without subjecting it to ammonium sulfate precipitation) to Sephacryl S-200 gel filtration (data not shown). These observations point to the possible association of the CaM-BP68 with macromolecular structure in the nuclear lysate of FDC-P1 cells. At pH 7.4 and 10 mM KCl, most of the CaM-binding proteins in the fractions with the CaM-BP68 are adsorbed to the DEAE anion-exchange cartridge. These proteins, particularly the CaM-BP68, are eluted at approximately 200 mM KCl (Figure 2).

The CaM-BP68 was retained on the CaM–agarose affinity column in the presence of the buffer with 2 mM Ca^{2+} and was specifically eluted with 10 mM EGTA (Figure 2). At this stage of purification, the CaM-BP68 has undergone an approximately 270-fold enrichment over its concentration in whole cell extracts (Table 1) and was the major silver-stained band on SDS-PAGE (data not shown). Furthermore, C_8 reversed-phase HPLC analysis of the CaM-BP68-containing fraction eluted from CaM-agarose column exhibited a single major peak that eluted with about 50% acetonitrile (data not shown). Although there is about 50% loss in the overall recovery of the protein following C_8 reversed-phase HPLC (data not shown), at this stage of purification, the CaM-BP68 is enriched by about 2400-fold as compared to the total cell extract (Table 1). As shown in Figure 4, the pooled protein fractions obtained following the application of nuclear lysate through the five sequential steps of the CaM-BP68 purification

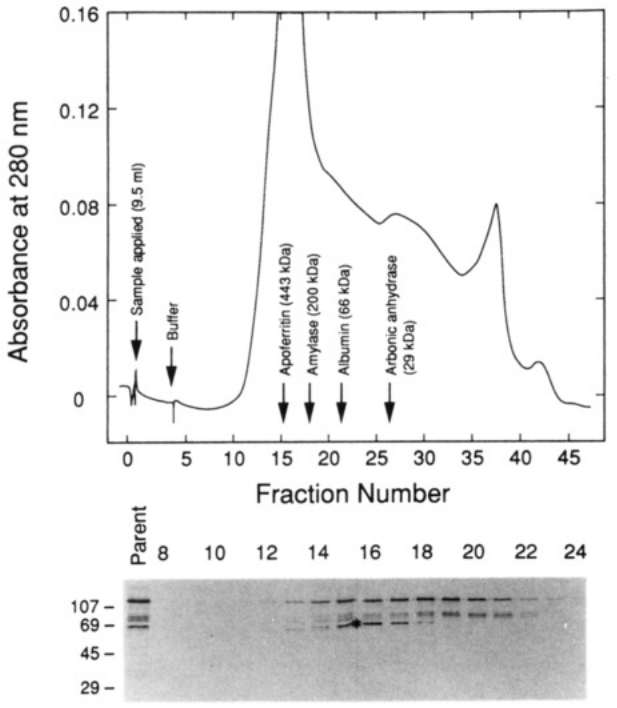
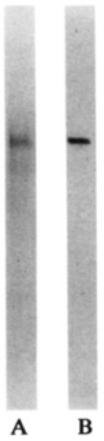


FIGURE 3: Sephacryl S-200 gel filtration of the 35% $AmSO_4$ precipitated fraction from nuclear lysate of FDC-P1 cells. The CaM-BP68-containing fraction obtained following 35% ammonium sulfate precipitation was applied to Sephacryl S-200 gel filtration column. The column was then developed with buffer B, individual fractions were collected, and each fraction was assayed for CaM-binding proteins as described under Materials and Methods. The top panel shows the elution profile of the material that exhibits absorbance at 280 nm, and the molecular weight marker proteins (indicated by arrows). Elution of marker proteins was determined by subjecting each of the marker proteins to gel filtration under the conditions employed for gel filtration of the dissolved 35% ammonium sulfate precipitate. The lower panel shows the profile of CaM-BPs in individual fractions. The asterisk indicates the position of the CaM-BP68.



A) Silver-staining
B) CaM-binding

FIGURE 4: SDS–PAGE analysis of purified CaM-BP68. Nuclear lysate of FDC-P1 cells was subjected to five sequential steps of CaM-BP68 purification as described in Figure 2. Proteins in CaM-BP68-containing fraction, obtained following C_8 reversed-phase HPLC, were subjected to SDS-PAGE and analyzed by silver staining (A) and CaM-binding assay (B) as described under Materials and Methods.

scheme exhibited essentially a single silver-stained protein band of about 68 kDa (lane A) with calmodulin-binding property (lane B) as determined by SDS–PAGE analysis.

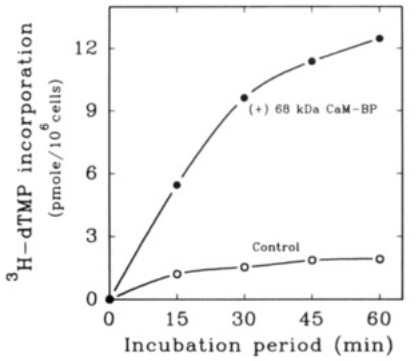


FIGURE 5: Effect of purified CaM-BP68 on [^{3}H]dTTP incorporation into DNA of permeabilized FDC-P1 cells. Log-growing FDC-P1 cells were permeabilized and the rate of DNA replication was determined as described under Materials and Methods. [^{3}H]dTMP incorporation in the presence of 60 μg/mL of either BSA (control) or purified CaM-BP68 is shown by open and solid circles, respectively. Data represents the mean of duplicate determinations in each of two experiments. Error bars smaller than the symbols represent standard deviation.

Effect of Purified CaM-BP68 on DNA Replication in Permeabilized FDC-P1 Cells. In earlier studies (Reddy et al., 1992a), it was observed that the nuclear localization of the CaM-BP68 is temporally correlated with IL-3-dependent progression of cells from G1 to S phase, suggesting its possible involvement in the onset of nuclear DNA replication. To evaluate this possibility, we have established a permeabilization procedure for FDC-P1 cells that allows monitoring of DNA synthesis in the presence of exogenous precursors and protein factors. This permeabilization method is similar to the one employed successfully in earlier studies to evaluate the effects of CaM-specific monoclonal antibodies on DNA replication in fibroblast cells (Reddy et al., 1992b). As in the case of fibroblast cells (Reddy et al., 1992b), lysolecithin treatment of FDC-P1 cells allowed the incorporation of exogenous deoxynucleotides into DNA at a linear rate for 10–15 min in the reaction mixture optimized for DNA synthesis (Figure 5). Corresponding to the presence of less than 20% of the cells in S phase in the cultures employed in these studies, a low-level incorporation of [^{3}H]dTTP was observed in the control incubation. However, when the purified CaM-BP was included in the reaction mixture, the overall incorporation of [^{3}H]dTMP was increased by 3–4-fold as compared to the controls (Figure 5). Furthermore, the rate of such incorporation was linear for a prolonged period of 30–40 min. This increase in DNA synthesis of permeabilized FDC-P1 cells was dependent on an increase in the concentration of the CaM-BP68 in the reaction mixture (Figure 6). Within the range of the concentrations of the CaM-BP68 examined, a direct correlation was observed between the amount of exogenously added protein and the rate of deoxynucleotide incorporation. The maximum increase in the rate of incorporation was approximately 4-fold above that of control cells (Figure 6). This increased incorporation rate was similar to that observed in permeabilized cultures with more than 75% of the cells in S phase obtained following synchronization by an isoleucine deprivation method (data not shown). The CaM-BP68-containing fraction itself did not exhibit any *in vitro* DNA polymerase α activity when measured by employing exogenous DNA primer/template (data not shown) as described elsewhere (Reddy & Pardee, 1980). These observations provide direct evidence for the involvement of the CaM-BP68 in DNA synthesis of hemopoietic progenitor cells.

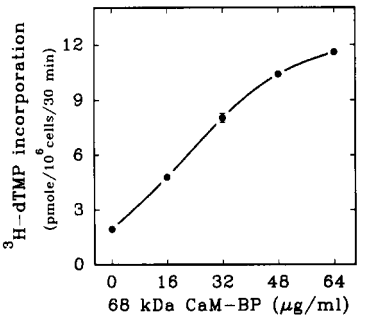


FIGURE 6: Effect of increasing concentrations of CaM-BP68 on [^{3}H]-dTTP incorporation into DNA of permeabilized FDC-P1 cells. Experimental procedures are as described in legend to Figure 9, except the amount of purified CaM-BP68 in each assay is as shown in the figure. Error bars smaller than the symbols represent standard deviation, $n = 4$.

DISCUSSION

How is the signal generated by growth factor–membrane receptor interactions transmitted to the nucleus to stimulate the onset of DNA replication (S phase)? The overall sequence of events in the signaling pathways leading to the onset of DNA synthesis remains the subject of intense investigation. The present study has identified a unique protein that is both modulated in direct response to proliferative growth factors and involved in the regulation of nuclear DNA replication in mammalian cells. We have demonstrated that the nuclear localization of a specific CaM-BP of approximately 68 kDa is dependent on the exposure of the hemopoietic progenitor cells to cytokines with proliferative potential. Moreover, this CaM-BP68 purified from a murine factor dependent cell line (FDC-P1) causes an increase in DNA synthesis in permeabilized cells. The cytokine-dependent modulation of the CaM-BP68 is observed in hematopoietic cells derived from both rodents (Figure 1A) and humans (Figure 1B). These observations, taken together, suggest that the growth factor dependent mitogenic stimulation of a variety of mammalian cells is regulated in part by a common mechanism involving nuclear localization of the CaM-BP68, which in turn regulates nuclear DNA synthesis.

CaM may play a pivotal role in the regulation of cell proliferation (Lu & Means, 1993; Means & Rasmussen, 1988; Whitfield et al., 1988). In recent years considerable effort has been expended studying the molecular mechanisms associated with Ca^{2+} and CaM dependent regulation of mitogenic stimulation in mammalian cells (Lu & Means, 1993). Intracellular Ca^{2+} and CaM, increased in the late G1 period prior to the onset of DNA synthesis (S phase), have been shown to translocate to the nucleus and associate with the nuclear matrix (Pujol et al., 1989; Serratosa et al., 1988). Nuclear matrix is suggested to be the site for newly replicating DNA and contains the enzymes of DNA synthesis assembled into complex (Nelson et al., 1986; Tubo & Berezney, 1987). More recently, it was demonstrated that CaM-specific monoclonal antibodies markedly inhibit DNA replication in permeabilized fibroblasts (Reddy et al., 1992b). Furthermore, calmodulin antagonists, such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), inhibit [^{3}H]-thymidine incorporation into DNA of FDC-P1 cells (Reddy et al., 1992a). These, and other (Chafouleas et al., 1984; Rasmussen & Means, 1987; Boynton et al., 1980), observations indicate that Ca^{2+} and CaM are involved in the regulation of nuclear DNA synthesis by interacting with the DNA replication machinery.

If CaM is involved in DNA replication, which specific enzyme(s) in the replication machinery depend on its (their) interaction with CaM for the activity? While several key DNA-synthesizing enzymes, including ribonucleotide reductase, thymidylate synthase, and DNA polymerase α, are known to require normal levels of Ca^{2+} and CaM in intact cells for their activity (Whitfield et al., 1982; Lopez-Girona et al., 1992), none of these enzymes isolated from the cells exhibits specific dependence on Ca^{2+} or CaM for its *in vitro* activity. DNA polymerase α, immunopurified from a variety of mammalian cells, contains specific CaM-binding proteins (Hammond et al., 1988), but its activity *in vitro* is unaffected by CaM or CaM antagonists. The isolated enzymes in their purified form may be stripped of accessory proteins critical for CaM-dependent regulation. Consistent with this possibility is the observation that in permeabilized cells monoclonal antibodies specific to CaM caused a dose-dependent inhibition of DNA replication without exhibiting an inhibitory effect on DNA polymerase activity measured in the soluble lysate prepared from the same cells (Reddy et al., 1992b). In a similar vein, the purified CaM-BP68 did not exhibit DNA polymerase activity when measured *in vitro* but caused dramatic stimulation of DNA synthesis in permeabilized log-growing FDC-P1 cells (Figures 5 and 6).

Cyclins, in association with specific protein kinases, have emerged as the primary regulators of cell cycle progression in eukaryotic cells (Sherr, 1993; D'Urso et al., 1990; Fang & Newport, 1991; Xiong et al., 1992; Lew et al., 1991). Cyclin E (Ohtsubo & Roberts, 1993) and cyclin D (Matsushime et al., 1991), in association with $p34^{cdc2}$ kinase-like proteins or cdk2 or cdk4 kinases, appear to regulate the progression of mammalian cells from G1 to S phase, while cyclin B, in association with $p34^{cdc2}$ kinase, is required for G2/M transition (Gould & Nurse, 1989). Ca^{2+} and CaM appear to be involved in the catalytic activation of $p34^{cdc2}$ kinase by activating a phosphatase responsible for dephosphorylation of tyrosine residue in the cdc2 kinase in *Aspergillus nidulous* (Lu & Means, 1993). Similar involvement of CaM in activation of cyclin E or cyclin D associated protein kinases remains to be determined. Although the cyclins and their associated protein kinases are essential for cell cycle progression, their involvement does not appear to be sufficient for growth factor dependent transition of cells from G1 to S phase (Ohtsubo & Roberts, 1993). It is possible that CaM-BP68, on the basis of both its stringent growth factor dependent modulation and its ability to cause an abrupt stimulation of DNA synthesis, may represent an important target for the cyclin–protein kinase complex stimulating G1/S transition in mammalian cells.

The CaM-BP68 is modulated in direct response to mitogenic stimulation by growth factors, suggesting that the addition of this CaM-BP to log-growing cells may have advanced the cells limited for this protein from G1 into S phase, thereby increasing the number of cells undergoing DNA synthesis. This hypothesis appears more likely if one envisions the possible involvement of CaM-BP68 in cyclin–protein kinase complexes. Alternatively, CaM-BP68 may stabilize the DNA replication machinery, thereby increasing ability to replicate DNA in permeabilized cells. The stabilizing function of CaM-BP68 would ensure that the deoxynucleotides are incorporated into DNA more efficiently and at a linear rate for a prolonged period. Irrespective of the specific mechanism, the present studies demonstrate that the calmodulin-binding property of a specific 68-kDa protein ismodulated in direct response to

cytokines with proliferative potential in a variety of hemopoietic progenitor cells and that the purified CaM-BP68 causes a dramatic increase in DNA replication in permeabilized FDC-P1 cells.

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