Calmodulin-Specific Monoclonal Antibodies Inhibit DNA Replication in Mammalian Cells†

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ABSTRACT: The involvement of calmodulin in the proliferation of Chinese hamster embryo fibroblast cells has been studied with a specific monoclonal antibody to calmodulin. We observed that calmodulin levels increase 2-fold in the late G1 period in these cells, and this coincides with the increase in DNA polymerase α activity as the cells progress synchronously from a quiescent state in the G1 to the S phase. However, there is a concurrent 10-fold enhancement of thymidine kinase activity, which is tightly coupled to the entry of cells into the S phase. Incubation of permeabilized S-phase cells with calmodulin-specific murine monoclonal antibody resulted in a dose-dependent inhibition of DNA replication. This inhibitory effect of anti-calmodulin antibodies on DNA replication is completely reversed by the addition of exogenously purified calmodulin. These observations provide evidence for the involvement of calmodulin in DNA replication and, therefore, in cell proliferation during the S phase.

The control of DNA replication in eukaryotes is poorly understood. An integrated role of Ca^{2+} and calmodulin (CaM),[1] the major intracellular Ca^{2+}-binding protein, in cell proliferation is widely implicated from a number of in vivo and in vitro observations (Boynton et al., 1980; Hidaka et al., 1981; MacManus et al., 1981; Means & Rasmussen, 1988; Rasmussen & Means, 1989; Veigl et al., 1984a). For example, inositol phosphate mediated release of Ca^{2+} from intracellular stores is known to stimulate the initiation of DNA synthesis (Hill et al., 1989), increased intracellular CaM reduces the length of the cell cycle by shortening the G1 phase (Rasmussen & Means, 1989), CaM content increases in regenerating liver (MacManus et al., 1981), and an increase in intracellular CaM levels in a variety of mammalian cells coincides with the initiation of DNA replication (Chafouleas et al., 1981, 1982; Pinol et al., 1988). However, it was also reported that although CaM levels double on a per cell basis, the concentration of CaM in relation to total soluble cell proteins remains unchanged as the cells progress from the G1 to the S phase (Veigl et al., 1982, 1984b). Furthermore, since CaM regulates a variety of biosynthetic processes in mammalian cells (Cheung, 1980; Klee et al., 1980; Klee & Vanaman, 1982; Wang et al., 1985; Means 1988), specific involvement of CaM in cell proliferation or DNA replication cannot be established solely from changes observed in CaM levels during the cell cycle. Similarly, pharmacological inhibitors of CaM, which have been used to obtain evidence for involvement of CaM in cell proliferation, are nonspecific and may influence CaM-independent events. Therefore, the specific involvement of CaM in cell proliferation, particularly as the cells progress through the S phase, remains to be established.

In order to delineate the role of CaM in cell proliferation, we compared the changes in CaM levels to the changes in the activities of the enzymes associated with DNA synthesis as fibroblast cells progress synchronously from the G1 to the S phase and examined the effect of CaM-specific monoclonal antibodies (Sacks et al., 1991) on DNA replication in permeabilized S-phase cells. These studies provide more direct evidence supporting the involvement of CaM in DNA synthesis and, therefore, in the progression of mammalian cells through the S phase.

MATERIALS AND METHODS

Cell Culture and Synchronization. Chinese hamster embryo fibroblast (CHEF/18) cells were routinely grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 6% fetal calf serum (Sigma Chemical Co.), 3 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 8% CO_2. These cultures were mycoplasma-free as tested by the method of Schneider et al. (1974). Cells grown in 35-mm or 150-mm Petri dishes to a density of (1–4) × 10^3 cells/10 mm^2 were synchronized by the isoleucine starvation method as described elsewhere (Reddy, 1989).

Measurement of [^{3}H]Thymidine Incorporation into DNA. At regular intervals after being released from the isoleucine block, cells grown in 35-mm Petri dishes were pulse labeled with [^{3}H]thymidine (5 μCi/mL) for 30 min. [^{3}H]Thymidine incorporated into DNA and nuclei were determined by liquid scintillation counting of acid-precipitable material and autoradiography of the labeled cells, respectively, as described (Reddy, 1989).

Preparation of Soluble Cell Lysate. Synchronized CHEF/18 cells in 150-mm Petri dishes were collected by trypsinization at regular intervals after being released from the isoleucine block. Following one wash with a buffer containing 35 mM Hepes (pH 7.4), 150 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$,

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[1] Abbreviations: CaM, calmodulin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; CHEF, Chinese hamster embryo fibroblast; GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; IL, interleukin.

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1 mM phenylmethanesulfonyl fluoride, and 8 mM dithiothreitol, the cells were suspended in the same buffer at a density of 5×10^7 cells/mL. The cells were subjected to ultrasonic disruption using a Branson sonifier 250 with a micro-tip at an output setting of 1.5 and a duty cycle of 10% for 30–40 pulses, the homogenate was clarified by centrifugation in an Eppendorf microfuge, and the supernatant was treated as the soluble cell lysate. Protein content in the fractions was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Calmodulin Measurement. Calmodulin levels in soluble cell lysates were determined by extracting CaM from individual samples as described by Pujol et al. (1989) and assaying in triplicate by radioimmunoassay with the anti-CaM monoclonal antibody as described (Sacks et al., 1991).

Determination of Thymidine and DNA Polymerase Activities. Thymidine kinase and DNA polymerase activities in individual lysates were determined as described by Subramanyam et al. (1990). Each value presented for these two enzymes is a mean of four determinations, and there was less than 10% variation among the determinations.

Permeabilization of S-Phase CHEF/18 Cells. CHEF/18 cells grown in 150-mm Petri dishes were synchronized by the isoleucine starvation method as described (Reddy, 1989). Ten to twelve hours after being released from the isoleucine block, cells were collected by trypsinization and permeabilized with L-α-lysophosphatidylcholine (lysolecithin) as described by Reddy and Pardee (1982).

Measurement of DNA Synthesis in Permeabilized S-Phase CHEF/18 Cells. Permeabilized S-phase cells (1.5×10^6 cells) were treated with anti-CaM or nonspecific monoclonal antibodies at room temperature for 3–4 min and incubated with a 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, and 25 μM [^{3}H]dTTP (110 cpm/pmol) in a final volume of 0.075 mL for 20 min at 37 °C. This reaction mixture produced maximal DNA replication in lysolecithin-treated S-phase CHEF/18 cells (Reddy & Pardee, 1982). Radioactivity incorporated into the acid-precipitable material was determined as described (Reddy & Pardee, 1982).

Immunocytochemical Staining of the Nuclei from Permeabilized Cells Treated with Anti-CaM or Nonspecific Monoclonal Antibodies. Permeabilized S-phase CHEF/18 cells treated with 0.25 nmol of anti-CaM or anti-myoglobin ($IgG_1 \kappa$) monoclonal antibodies, or with an equivalent volume of PBS (control), were incubated with the DNA replication reaction mixture as described above and washed twice with 1.5 mL of PBS by centrifugation at 3000 rpm for 10 min. Cells were then gently suspended in 1 mL of PBS, and a 100-μL aliquot was subjected to Cytospin (Shandon). Nuclei of these cells transferred to the slides were fixed in 1% (v/v) glutaraldehyde, washed three times for 5 min each with PBS, and incubated with 5% (w/v) nonfat dry milk in PBS for 30 min. Slides were then treated with biotin-conjugated anti-mouse IgG (1:1000 diluted, Sigma) for 2 h followed by three washes of 5 min each with PBS. These slides were then incubated for 2 h with alkaline phosphatase-conjugated streptavidin (1:2000 diluted, Calbiochem Corp., San Diego, CA), washed three times with PBS, and stained with nitroblue tetrazolium. Stained nuclei were mounted with a cover slip using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA), and photomicrographs of representative fields were obtained by employing a camera attached to a Leitz Laborlux 12 light microscope.

RESULTS AND DISCUSSION

We have initially attempted to understand the significance of CaM in cellular proliferation by comparing its levels in the cell cycle to the activities of two classic enzymes related to DNA synthesis. These are DNA polymerase α, whose activity is essential for DNA replication, and thymidine kinase, whose expression is tightly coupled to the progression of cells from the G1 to the S phase (Coppock & Pardee, 1987). As shown in Figure 1A, Chinese hamster embryo fibroblast (CHEF/18) cells synchronized by the isoleucine starvation method progress synchronously from a quiescent state in the G1 to the S phase. In these cells thymidine kinase activity increases approximately 10-fold (Figure 1B), which is almost identical to the extent of the increase in [^{3}H]thymidine incorporation into DNA (Figure 1A). This is evident when thymidine kinase activity is expressed both on a per cell basis and in relation to the total soluble protein, indicating its correlation with the onset of DNA synthesis. By contrast, DNA polymerase activity when expressed on a per cell basis exhibits a 1.5–2-fold increase during the same period, and this change becomes insignificant when corrected for total soluble cellular protein (Figure 1C). The effect of cell cycle progression on CaM levels (Figure 1D) is similar to that observed for DNA polymerase activity (Figure 1C), exhibiting a 2-fold increase or no change when expressed per cell or per milligram of soluble protein, respectively. These observations indicate that, unlike the specific increase in thymidine kinase activity, increases in DNA polymerase activity and CaM levels may not be specific to the progression of cells from the G1 to the S phase; rather they appear to be expressed in concert with many other cellular proteins during this period, and the extent of changes observed in the total cellular levels of some of the enzymes, such as DNA polymerase, and/or proteins, such as CaM, during the G1 phase may not signify their true role in DNA replication (S phase). Therefore, we employed a more direct approach and examined the effect of recently developed monoclonal antibodies that are highly specific to CaM (Sacks et al., 1991) on DNA replication in S-phase CHEF/18 cells.

Mammalian cells permeabilized by lysolecithin treatment are suitable for monitoring the rate of DNA replication from exogenous deoxynucleoside triphosphates (Castellot et al., 1978; Reddy & Pardee, 1982) and are also ideal for analyzing the roles of various DNA polymerases in nuclear DNA replication (Miller et al., 1985). The latter studies have been performed with individual polymerase-specific monoclonal antibodies (Miller et al., 1985) or inhibitors (Hammond et al., 1989). The suitability of lysolecithin-permeabilized S-phase CHEF/18 cells for examining the effects of anti-CaM antibody on nuclear DNA replication is supported by our observations that intranuclear CaM is readily accessible to these antibodies. This was demonstrated by immunocytochemical staining of the nuclei isolated from permeabilized S-phase cells exposed to anti-CaM monoclonal antibodies in the presence of the DNA replication reaction mixture (Figure 2). The nuclei of permeabilized cells incubated with anti-CaM antibody exhibit prominent immunocytochemical staining (Figure 2A), while no staining is detected in the cells incubated in the presence of either PBS alone (Figure 2B) or a nonspecific mouse monoclonal antibody (Figure 2C).

These cells and the antibodies were then employed to determine the involvement of CaM in nuclear DNA replication. As shown in Figure 3, we observed that increasing

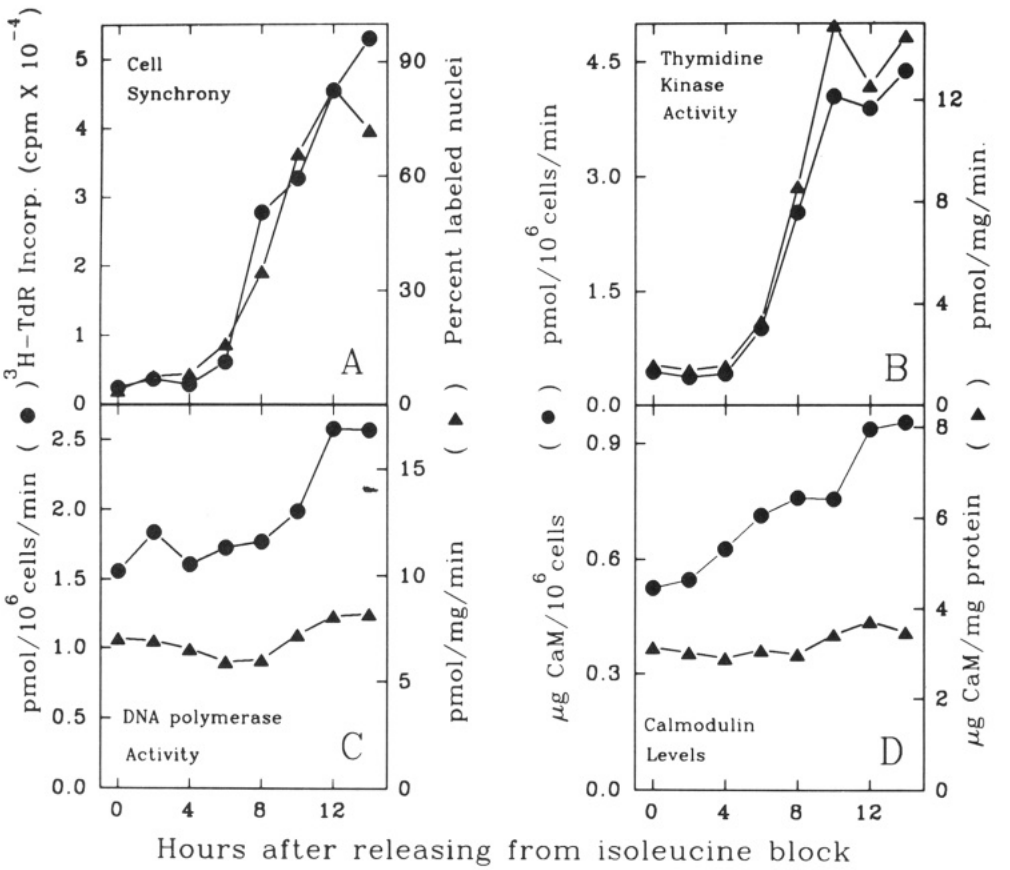

FIGURE 1: Changes in DNA polymerase and thymidine kinase activities and CaM levels as synchronized CHEF/18 cells progressed from the G1 to S phase. CHEF/18 cells were synchronized by the isoleucine starvation method, and at regular intervals after release from the isoleucine block, the ability of the cells to incorporate [^{3}H]thymidine into DNA (panel A) was determined. Soluble cell lysates prepared from the cells at the same intervals were used to measure the levels of thymidine kinase activity (panel B), DNA polymerase activity (panel C), and CaM (panel D). Experimental procedures are as described under Materials and Methods.

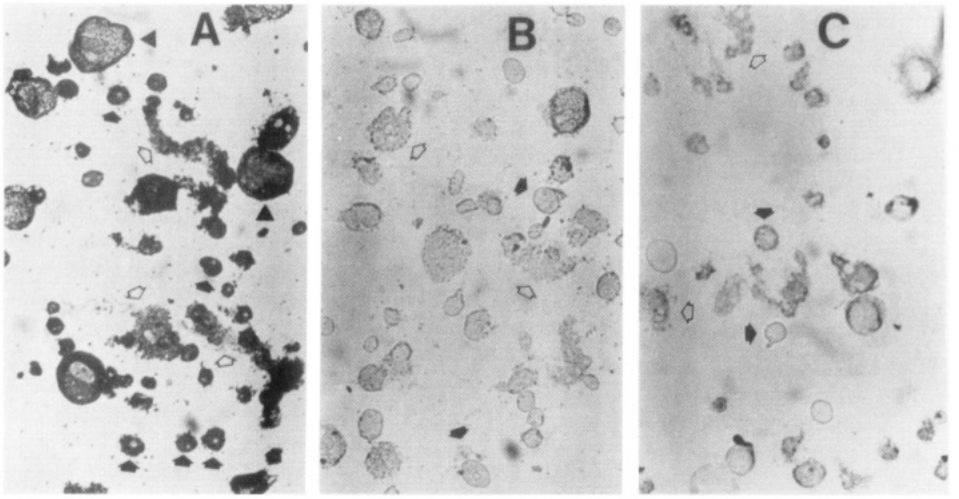

FIGURE 2: Immunocytochemical staining of nuclei from permeabilized S-phase CHEF/18 cells treated with anti-CaM monoclonal antibodies. Permeabilized S-phase CHEF/18 cells were incubated in a DNA replication reaction mixture in the presence of 0.25 nmol of anti-CaM monoclonal antibody (panel A), PBS (panel B), or 0.25 nmol of anti-myoglobin monoclonal antibody (panel C), and immunocytochemically stained preparations were recorded as described under Materials and Methods. Cells permeabilized with lysolecithin are fragile and fragment during the centrifugation required for the attachment of cells to the slide using Cytospin (Shandon). This procedure results in the retention of a large number of nuclei on the slide (indicated by closed arrows) with a background consisting largely of cytoplasm (indicated by open arrows). Occasionally, partially permeabilized cells that remained morphologically intact and exhibited both cytoplasmic and nuclear staining were observed in anti-CaM monoclonal antibody treated cells (indicated by closed triangles in panel A). The final magnification of the objects in each micrograph is 1000×.

concentrations of anti-CaM monoclonal antibodies inhibit, in a dose-dependent manner, the incorporation of [^{3}H]dTTP into DNA. The very high intracellular CaM concentrations (0.01–0.1 mM) necessitate the use of large amounts of antibody to block CaM-dependent processes (Cohen & Klee, 1988). In contrast, the anti-CaM antibodies do not inhibit DNA polymerase α activity in an in vitro assay performed with soluble cell lysates (data not shown), suggesting that these antibodies do not interact directly with the enzyme. In this permeabilized cell system DNA topoisomerase II (Nelson et al., 1986) antibodies or butylphenyldeoxyguanosine triphosphate, an inhibitor of DNA polymerase α and δ activities

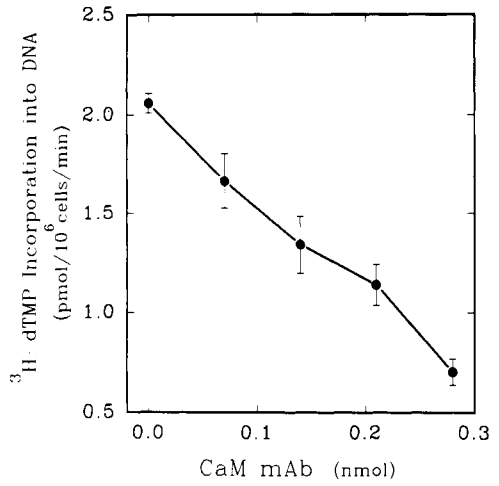

FIGURE 3: Effect of anti-calmodulin monoclonal antibodies on DNA replication in permeabilized S-phase CHEF/18 cells. Incorporation of [^{3}H]dTTP into DNA of permeabilized S-phase cells in the presence of increasing concentrations of anti-CaM monoclonal antibody (CaM mAb) was determined as described under Materials and Methods. Error bars are the standard deviation; n = 4.

Table I: Effect of Various Inhibitors of [^{3}H]dTTP Incorporation into DNA[a]

condition	pmol (10^6 cells)$^{-1}$ min^{-1}	%[b]
(A) control (no addition)	2.05	100
+nonimmune serum	2.12	103
+hsp70 monoclonal antibodies	1.85	90
+DNA topo II antibodies	0.13	6
+BuPhdGTP	0.05	2.5
+calmodulin antibodies	0.37	18
(B) control (no addition)	2.56	100
+myoglobin Ab (IgG$_1$ κ)	2.09	81

[a] Experimental procedures are as described under Materials and Methods. The DNA replication reaction mixture contained no additions (control), nonimmune serum (0.35 mg/mL), heat shock protein (hsp70)-specific monoclonal antibodies (C92) (12 μL, obtained from Dr. William J. Welch, University of California, San Francisco, CA), DNA topoisomerase II antibody (12 μL, obtained from Dr. Leroy F. Liu, Johns Hopkins University, Baltimore, MD), 0.3 mM butylphenyldeoxyguanosine triphosphate (BuPhdGTP), calmodulin monoclonal antibody (0.25 nmol), or myoglobin monoclonal antibody (0.35 nmol, obtained from Dr. Jack Ladenson, Washington University School of Medicine, St. Louis, MO). Experiments in steps A and B were performed on different days. The difference in control values is within routine interassay variability. Each value represents the average of duplicate determinations, and the variation between determinations is less than 10%. [b] Activity is expressed as a percentage of the activity obtained with no addition (control).

(Hammond et al., 1987), produced inhibitory effects similar to that observed with anti-CaM antibody (Table I). By contrast, as shown in Table I, nonimmune serum or monoclonal antibodies against the 70-kDa heat shock protein (hsp70), a nuclear protein (Welch, 1990) that has no reported role in DNA replication, did not significantly inhibit [^{3}H]dTTP incorporation. Furthermore, since the anti-CaM monoclonal antibody employed in these studies is mouse IgG$_1$ κ (Sacks et al., 1991), we have tested the effect of a nonspecific mouse IgG$_1$ κ antibody on [^{3}H]dTTP incorporation. As shown in Table I, the decrease in [^{3}H]dTTP incorporation is less than 20% in the presence of 0.35 nmol of nonspecific (anti-myoglobin) antibody, while 0.25 nmol of anti-CaM antibody produces an 80% decrease. This suggests that the inhibition produced by the anti-CaM antibody is due to specific binding to CaM, and not to a nonspecific effect of monoclonal antibodies. In this permeabilized cell system significant inhibition of [^{3}H]dTTP incorporation is observed only with antibodies directed against enzymes and proteins integrally

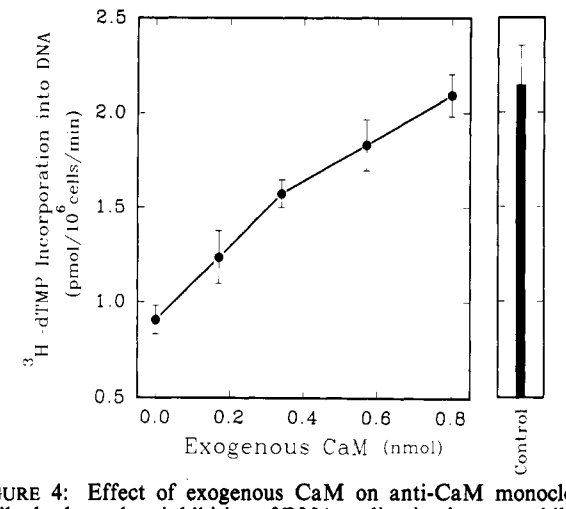

FIGURE 4: Effect of exogenous CaM on anti-CaM monoclonal antibody-dependent inhibition of DNA replication in permeabilized S-phase CHEF/18 cells. Permeabilized S-phase cells exposed to 0.25 nmol of anti-CaM monoclonal antibody were treated with increasing concentrations of CaM prior to incubation with the DNA replication reaction mixture. Control represents incubation without antibody or exogenous CaM. Experimental procedures are as described under Materials and Methods. Error bars are the standard deviation; n = 4.

involved in DNA replication. Thus, our observation that nuclear DNA replication in permeabilized S-phase CHEF/18 cells is almost as sensitive to anti-CaM monoclonal antibodies as it is to antibodies or an antimetabolite specific to the enzymes of DNA replication points to an important role for CaM in the control of DNA replication.

The specificity of an inhibitor can be evaluated by adding the target molecule to overcome inhibition. We, therefore, examined the effect of exogenous CaM on anti-CaM antibody-dependent inhibition of DNA replication. As shown in Figure 4, the inhibitory effect of anti-CaM monoclonal antibodies on DNA replication is completely reversed by the addition of increasing concentrations of purified CaM in the incubation mixture. Further confirmation is provided by adding exogenous CaM in the presence of the nonspecific anti-myoglobin antibody. This does not alter the small (less than 20%) decrease in DNA replication produced by the nonspecific antibody (data not shown). These observations strongly suggest that the inhibitory effect of anti-CaM monoclonal antibodies on [^{3}H]dTTP incorporation in permeabilized S-phase cells is due to their interaction with CaM associated with the replication machinery. Although it is difficult to definitively exclude the possibility that anti-CaM antibodies may recognize cellular proteins other than CaM, the anti-CaM monoclonal antibodies employed in these studies exhibit no cross-reactivity with other Ca^{2+}-binding proteins, which have significant sequence homology with CaM (Sacks et al., 1991), nor has cross-reactivity been observed with other cellular proteins (Sacks et al., 1992).

The involvement of CaM in DNA replication, inferred from the present studies, is consistent both with previous observations of proliferative activation-dependent association of CaM with the nuclear matrix (Pujol et al., 1989; Serratosa et al., 1988) [a suggested subcellular site for eukaryotic DNA replication (Nelson et al., 1986; Pardoll et al., 1980; Tubo & Berezney, 1987)] and with studies demonstrating the association of CaM with active DNA (Bachs & Carafoli, 1987). There is evidence to suggest that Ca^{2+} and CaM participate in the early postreceptor events in the cellular mechanism of insulin action (Graves et al., 1986; Sacks et al., 1992; Sacks & McDonald, 1988) that stimulate the entry of mammalian cells from the

G1 into the S phase (Campisi & Pardee, 1984). In addition, recent evidence suggests a role for CaM-binding proteins in DNA synthesis. A 68-kDa CaM-binding protein, associated with enzymes of DNA replication, translocates to the nucleus during both insulin-induced DNA synthesis (S phase) (Subramanyam et al., 1990) and proliferative stimulation of hemopoietic cells by growth factors (Reddy et al., 1992). Furthermore, specific CaM-binding proteins are tightly bound to immunopurified preparations of DNA polymerase α from a variety of mammalian cells (Hammond et al., 1988). During the preparation of this paper, Lopez-Girona et al. (1992) demonstrated that two anti-CaM drugs block the entry of quiescent NRK cells into the S phase by inhibiting the increase in DNA polymerase α activity.

Our data suggest that anti-CaM antibodies inhibit DNA replication in permeabilized cells by binding to CaM. However, purified preparations of DNA polymerase α are insensitive to CaM antagonists (Lopez-Girona et al., 1992) or anti-CaM antibodies. On the basis of these observations it is possible that specific CaM-binding proteins associated with DNA polymerase(s) (Hammond et al., 1988; Subramanyam et al., 1990) and modulated in specific response to growth factor-dependent proliferative stimulation (Reddy et al., 1992; Subramanyam et al., 1990) may play a role in CaM-directed regulation of DNA replication in intact cells. Further characterization of such CaM-binding proteins associated with the enzymes of DNA replication may prove to be critical in our understanding of the precise mechanism by which CaM controls cell proliferation.

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