Effect upon Mitogenic Stimulation of Calcium-dependent Phosphorylation of Cytoskeleton-associated 350,000- and 80,000-mol-wt Polypeptides in Quiescent 3Y1 Cells

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ABSTRACT Rabbit antiserum raised against highest molecular weight microtubule-associated protein (MAP-1) of brain immunoprecipitated 350,000-, 300,000-, and 80,000-mol-wt phosphoproteins of rat embryo fibroblasts (3Y1-B). The 350,000-mol-wt protein was sensitive to heat as was brain MAP-1, but the 300,000- and 80,000-mol-wt proteins were not. These polypeptides were hardly phosphorylated in cells in the quiescent G_0 phase but were rapidly phosphorylated after addition of serum, epidermal growth factor, phorbol ester, insulin, or transferrin in the presence of calcium ions. All these agents also induced incorporation of [^{3}H]-thymidine into DNA. These polypeptides were detected in isolated microtubules and cold-resistant filaments by immunoblotting. Since the 350,000-mol-wt polypeptide was detected in the membrane, the cytoskeletons, and the nucleus, and has been suggested to function as a linker, its rapid phosphorylation might represent an early process in transduction of the signal of mitogenic stimulation to the nucleus.

Microtubule-associated protein-1 (MAP-1)[1] is the highest molecular weight protein (M_r 340,000–370,000) that co-polymerizes with microtubules of the brain (1). This protein promotes the in vitro assembly of microtubules (2) and projects from the surface of the microtubules in a periodic manner (3). Immunofluorescent staining of the cytoplasmic network and mitotic spindle (4, 5, 6, 7, 8) and the centrosome (7, 9) with antisera and monoclonal antibodies against MAP-1 have suggested the association of the cross-reactive molecules with microtubules in the cell. Moreover, the high molecular weight polypeptides have been co-purified both with microtubules and with intermediate filaments (10). These results suggest a dual role of the polypeptides in the cell as regulators of microtubule assembly and as linkers between microtubules and intermediate filaments (6, 10). In addition, the high molecular weight peptides were phosphorylated both in vitro and in the cell (1, 10, 11), and their amount in pheochromocytoma cells increased upon treatment with nerve growth factor (12). The presence of the high molecular weight phosphoprotein in the plasma membrane suggests that it also has a role in the interaction of membrane proteins and microtubules (11). Cyclic AMP-dependent phosphorylation of the brain 300,000-mol-wt protein, MAP-2, is known to cause a drastic change in its interaction with actin filaments in vitro (13, 14).

We assumed that cellular analogues of MAP-1 act as linkers between receptors for growth factors and cytoskeletal components, and that their extent of phosphorylation regulates these interactions. We report the rapid Ca^{++}-dependent phosphorylations of 350,000- and 80,000-mol-wt polypeptides in response to various growth factors.

MATERIALS AND METHODS

Materials: The following materials were obtained from the people or manufacturers cited herein: 3Y1-B cells were a gift from Professor G. Kimura (Kyushu University); porcine brain microtubules were a gift from Dr. R.

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; FCS, fetal calf serum; MAP, microtubule-associated protein; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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Kuriyama (Wisconsin University); fetal calf serum (FCS) was obtained from Gibco Laboratories (Grand Island, NY); and phenylmethylsulfonyl fluoride was obtained from Boehringer Mannheim (Federal Republic of Germany). Leupeptin was obtained from Peptide Institute, Inc. (Minoo); and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), transferrin, and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, MO). Epidermal growth factor (EGF) was obtained from Toyobo Co. (Osaka); insulin from Fluka A.G. (Basel, Switzerland); EGTA from Nakari Chemicals Ltd. (Kyoto); PIPES from Dojin Chemical Inst. (Kumamoto); and GTP from Biochem. Indust. (Tokyo).

Cells and Culture Medium: We used clone 1-6 of 3Y1-B cells derived from Fischer rat embryo fibroblasts. This cell line shows growth inhibition on cell contact or serum deprivation (15). We cultured the cells in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% or 1% FCS in a CO_2-incubator.

Preparation of Antiserum: We purified MAP-1 from fresh rat brain by the rapid method previously described (16). Briefly, we isolated microtubule proteins by two cycles of temperature-dependent assembly and disassembly (17), and then separated MAPs from tubulin by DEAE-cellulose column chromatography. We then fractionated the MAP preparations by high pressure liquid chromatography on TSKOGEL G4000SW. The fraction with the highest molecular weight contained predominantly MAP-1, as shown by SDS PAGE, and was used as the immunogen. Antiserum was raised in rabbits by 12 repeated injections of this MAP-1 preparation intracutaneously into 100 sites each time every 2 wk.

Immunoblotting: We prepared brain microtubules, a whole cell extract, and cytoskeletons as samples for immunoblotting. We purified brain microtubules from porcine brain by two cycles of reversible assembly (17). We dissolved the whole cell pellet with sonication in hot SDS sample buffer (2% SDS and 5% 2-mercaptoethanol in Tris-HCl [pH 6.8], supplemented with 6 M urea) within 5 s to avoid proteolytic degradation. We isolated cytoskeletons from 3Y1-B cells by using Taxol (National Cancer Institute) (18). For this, we homogenized ~2 × 10^8 3Y1-B cells in 2 vol of extraction medium (pH 6.8) with protease inhibitors (0.1 M PIPES, 1 mM $MgCl_2$, 2 mM EGTA, 4 mM 2-mercaptoethanol, 0.9 M glycerine, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin, and centrifuged the cells at 50,000 *g* for 30 min. We incubated the supernatant with 20 μM Taxol and 1 mM GTP for 10 min at 37°C to allow the assembly of cytoskeletons. We then centrifuged the mixture at 50,000 *g* for 20 min through a layer of 10% sucrose in extraction medium containing 20 μM Taxol and 1 mM GTP. We used the precipitate, of which tubulin and actin were major components, as whole cytoskeletons. To isolate microtubules from other cytoskeletons, we centrifuged the first 50,000 *g* supernatant at 136,000 *g* for 60 min to precipitate cold-resistant filaments. Then we polymerized the microtubule proteins in the supernatant with Taxol and GTP. We separated these samples by electrophoresis on a linear gradient (4–15%) polyacrylamide gel. We electrophoretically transferred proteins to a nitrocellulose membrane, and stained them with immunoperoxidase as previously described (7).

Immunoprecipitation: We used an indirect immunoprecipitation method (19). Briefly, we lysed ~10^6 ^{32}P-labeled cells with 200 μl of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl and 0.05 M Tris-HCl [pH 8.0]) containing protease inhibitors, and clarified them by centrifugation at 14,000 rpm for 20 min. The supernatant was reacted with 15 μl of antiserum for 30 min at room temperature; we then added 45 μl of a 10% suspension of *Staphylococcus aureus* Cowan 1 to adsorb the immune complexes. After 10 min at room temperature, the sample was washed three times with lysis buffer and processed for 4–15% SDS PAGE. We stained and dried the gel for autoradiography with Kodak X-Omat AR film. After development of the film, we counted the radioactivities of gel slices corresponding to the 350,000- and 80,000-mol-wt bands in toluene scintillator in a Beckman liquid scintillation counter.

RESULTS

Molecules with Immunoreactivity with the Antiserum

We raised rabbit antiserum against purified MAP-1 prepared from rat brain microtubules. We determined the immunoreactive molecules of brain microtubules or of 3Y1-B cells by immunoblotting, as shown in Fig. 1. We examined brain microtubules (*A*, *a*), the whole cell extract (*B*, *b*), and whole cytoskeletons, which were prepared from 3Y1-B cells using Taxol and GTP (*C*, *c*). Tubulin and actin were the major components of the cytoskeletal preparation. The patterns of Coomassie blue staining of polyacrylamide gel after electrophoresis (*A*, *B*, *C*) were compared with immunoperoxidase staining of blotted membranes (*a*, *b*, *c*). The antiserum reacted with three bands of MAP-1 (M_r 355,000, 345,000, and 325,000) and MAP-2 (M_r 300,000) but not with other microtubular proteins of the brain. The three bands of highest molecular weights correspond to MAP-1-A,B,C named by Bloom et al. (8). The major cross-reactive polypeptides of the whole cell extract and cytoskeletons were the 350,000-, 300,000-, 80,000-, and 68,000-mol-wt proteins. Minor bands of cross-reactivity were occasionally seen at molecular weights of 290,000–200,000, 51,000–74,000, and 18,000–30,000. The cytoskeletons were further separated into microtubules and cold-resistant filaments. We then boiled an aliquot of the microtubule preparation for 5 min in the presence of 0.6 M NaCl to examine the heat sensitivity of the antigenic molecules. Fig. 2 shows the Coomassie Blue staining of SDS gels (*A–E*) and the immunoperoxidase staining of blotted membranes (*b–e*). The 350,000-mol-wt band on immunoblotting was less evident in microtubules than in cold-resistant filaments. The 350,000-mol-wt polypeptide was denatured by boiling and was detected in the pellet obtained by centrifugation of the heat-treated preparation. On the other hand, the 300,000, 80,000-, and 68,000-mol-wt polypeptides were heat-resistant, and remained in the supernatant. With regard to heat sensitivity, the 350,000-mol-wt polypeptide is like brain MAP-1 and the 300,000-mol-wt polypeptide is like brain MAP-2.

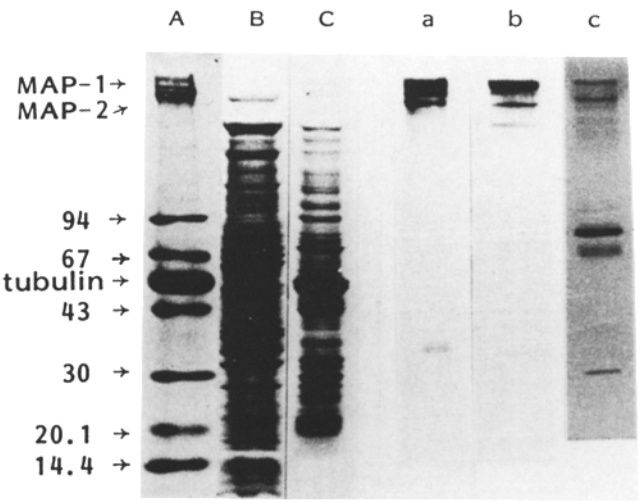

FIGURE 1 Immunoblotting of brain microtubule proteins and molecular weight markers (*A* and *a*), whole cell extract (*B* and *b*), and whole cytoskeletons that were prepared from 3Y1-B cells using Taxol and GTP (C and c). Comparisons of the patterns of Coomassie Blue stain (*A–C*) and immunoperoxidase staining (a–c) indicate that the main immunoreactive molecules have molecular weights of 355,000, 345,000, and 325,000 (MAP-1) (*A–C*) and 300,000 (MAP-2) in the brain, and 350,000, 300,000, 80,000, and 68,000, with a few polypeptides of lower molecular weights, in 3Y1-B cells.

Relation of Phosphorylation to the Cell Cycle

In medium containing 1% FCS, 3Y1-B cells ceased to proliferate on the second day of culture and thereafter remained in the quiescent stage. Addition of fresh serum to the quiescent cells induced DNA synthesis after a latent period of

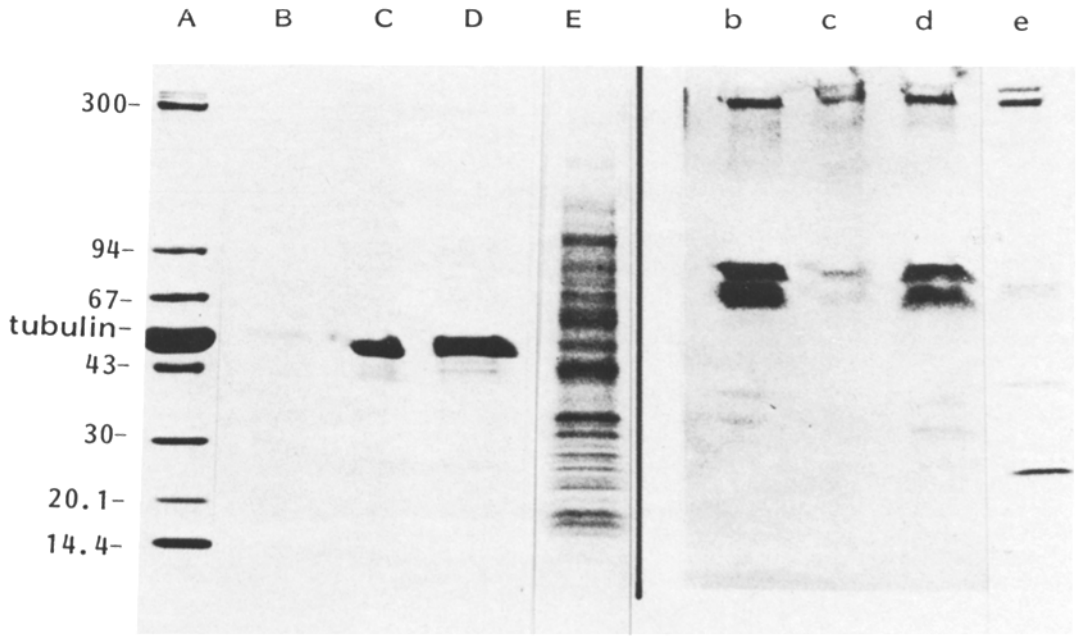

FIGURE 2 Immunoblotting to detect cross-reactive molecules in cytoskeletons and their heat sensitivity. About 2×10^8 3Y1-B cells were homogenized in 2 vol of 0.1 M PIPES buffer (pH 6.8) containing 2 mM EGTA, 1 mM $MgSO_4$, 0.9 M glycerol, and protease inhibitors. The 50,000 *g* supernatant was further centrifuged at 136,000 *g* for 60 min to precipitate cold-resistant filaments (*E* and e). Microtubules in the last supernatant were precipitated after polymerization with 20 μM Taxol and 1 mM GTP (*D* and *d*). The cellular microtubule preparation was heated at 100°C for 5 min in the presence of 0.6 M NaCl, and centrifuged to separate heat-sensitive proteins (*C* and *c*) from heat-resistant proteins in the supernatant (*B* and *b*). *A* shows brain microtubule proteins and molecular weight markers. Comparison of Coomassie Blue-stained gel (*A–E*) with immunoperoxidase-stained membrane (*b–e*) indicates that both cold-resistant filaments and microtubules contain 350,000 and 80,000-mol-wt polypeptides, and that the 350,000-mol-wt protein is denatured by boiling as is brain MAP-1.

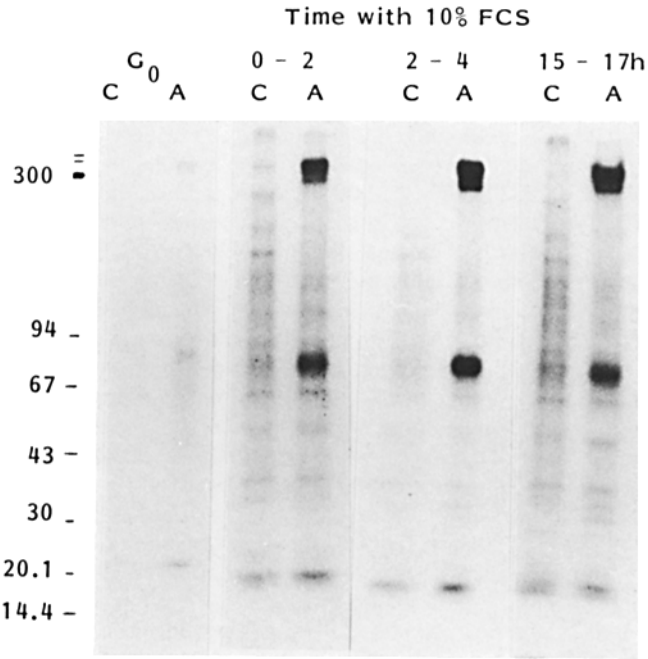

FIGURE 3 Autoradiographs of ^{32}P-phosphoproteins in quiescent (G_0-phase) 3Y1-B cells and in the stimulated cells 0–2 h ($G_0 \rightarrow G_1$), 2–4 h (G_1-phase), and 15–17 h (S-phase) after the addition of fresh serum. After incubation for 2 h with 50 μCi ^{32}P-phosphorus in P-free medium, cellular proteins in the lysis buffer were immunoprecipitated with anti-MAP-1 serum (*A*) or normal rabbit serum (*C*) and *Staphylococcus aureus* Cowan 1.

~10 h. Time-course experiments showed that the peak of DNA synthesis occurred at 16 h and the peak of mitosis at 20 h after stimulation (15, 20). Cells at different stages of the cell cycle were incubated in phosphate-free minimal essential medium containing 50 μCi/ml of carrier-free ^{32}P-phosphorus for 2 h. Extract of the labeled cells in lysis buffer were subjected to immunoprecipitation with anti-MAP-1 antiserum or with normal rabbit serum.

Fig. 3 shows autoradiograms of the immunoprecipitated proteins after their electrophoresis in 4–15% polyacrylamide gels. The major phosphoproteins that reacted with anti-MAP-1 serum but not with normal rabbit serum were the 350,000-, 300,000-, and 80,000-mol-wt proteins. These polypeptides were scarcely phosphorylated when ^{32}P-phosphorus was added to quiescent cells (Fig. 3*A*). When ^{32}P was added immediately after stimulation with serum, labeling of these polypeptides during the next 2 h increased fivefold. The phosphorylation increased further when ^{32}P was added from 2 to 4 h after stimulation. S-Phase cells (15–17 h after stimulation) also showed enhanced labeling of the polypeptides (Fig. 3*C*). Phosphorylation of the 350,000-mol-wt band increased in parallel with that of the 80,000-mol-wt band.

Effects of Growth Factors

We used two procedures to examine the effects of growth factors on quiescent 3Y1-B cells. One was the simultaneous application of various growth factors and ^{32}P for 2 h (Fig. 4). The other was the incorporation of ^{32}P into quiescent cells for 16 h, and the subsequent application of growth factors for 20

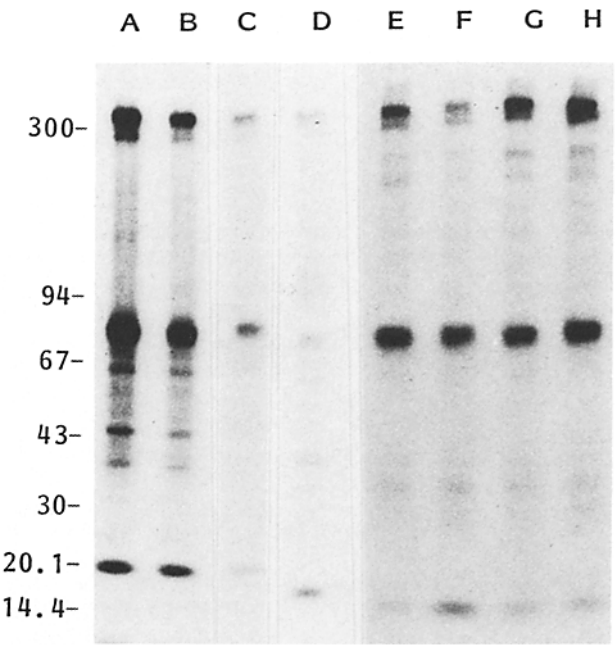

FIGURE 4 Enhancement of phosphorylation of 350,000- and 80,000-mol-wt proteins after mitogenic stimulation. Fresh FCS (10%) (*A*), TPA (0.1 μg/ml) (*B*), TPA (0.1 μg/ml) + EGTA (3 mM) (*C*), no treatment (*D*), EGF (30 ng/ml) (*E*), EGF (30 ng/ml) + EGTA (3 mM) (*F*), insulin (1 μg/ml) (*G*), and transferrin (10 μg/ml) (*H*) were added to serum-starved quiescent 3Y1-B cells and incubated with 50 μCi ^{32}P for 2 h. Labeled phosphoproteins were immunoprecipitated with anti-MAP-1 serum, and then resolved by 4–15% SDS PAGE for autoradiography.

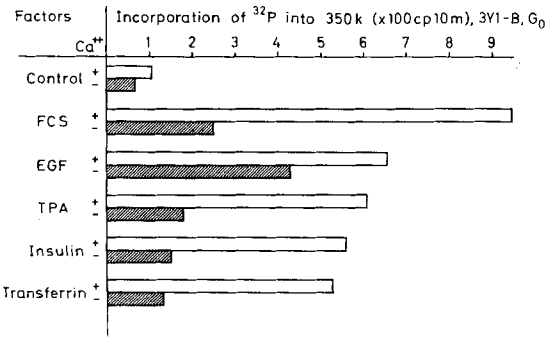

FIGURE 5 Radioactivities of slices of gels containing 350,000-mol-wt ^{32}P-phosphoproteins, measured in toluene scintillator in a Beckman liquid scintillation counter. Various factors were added to quiescent 3Y1-B cells in the presence (Ca^{++}–) or absence (Ca^{++}+) of 3 mM EGTA. Experimental conditions were as for Fig. 4. Mean values of three independent experiments are shown.

min. The latter procedure was designed to prelabel the ATP pool for detection of rapid phosphorylation and to avoid the complication of possible change in permeability of ^{32}P induced by the growth factors. Both procedures demonstrated that FCS (10%), EGF (30 ng/ml), TPA (0.1 μg/ml), insulin (1 μg/ml), and transferrin (10 μg/ml) stimulated the phosphorylations of 350,000- and 80,000-mol-wt polypeptides in the presence of Ca^{++}. Application of 3 mM EGTA to chelate Ca^{++} 5 min before the addition of the growth factors suppressed the stimulation of phosphorylation (Fig. 5).

Table I shows the radioactivity of the excised piece of gel containing the 350,000-mol-wt polypeptide. The first column represents the results of the first experimental procedure (simultaneous additions of factors and ^{32}P) and the second column represents the results of the other procedure (previous incorporation of ^{32}P). The ratios of increase in phosphorylation of the 350,000-mol-wt polypeptide and of the 80,000-mol-wt polypeptide were parallel.

To examine the relationship between phosphorylation of these polypeptides and mitogenic stimulation, we determined the incorporation of [^{3}H]thymidine into the acid-insoluble fraction. Quiescent cells were stimulated by growth factors with or without 3 mM EGTA, and then incubated with [^{3}H]thymidine from 5 to 20 h after stimulation. As indicated in Table I, the factors that stimulated the phosphorylations of the 350,000- and 80,000-mol-wt proteins (EGF, TPA, insulin, and transferrin) also stimulated DNA synthesis. On the other hand, addition of EGTA with these growth factors or hydrocortisone resulted in no increase in phosphorylation of the polypeptides or incorporation of [^{3}H]thymidine into DNA.

TABLE I
Effects of Factors on Quiescent 3Y1-B Cells

Treatment	Incorporation of ^{32}P into 350,000-mol-wt protein		Incorporation of [^{3}H]TdR into acid-insoluble fraction
	Procedure 1	Procedure 2	
	cp10m	*cp10m*	*cpm*
None	105 (1)*	210 (1)*	5,740 (1)*
EGTA (3 mM)	67 (0.63)	104 (0.50)	1,952 (0.34)
FCS (10%)	945 (9.0)	2772 (13.2)	175,644 (30.6)
EGTA + FCS	250 (2.4)	286 (1.36)	5,338 (0.93)
EGF (30 ng/ml)	656 (6.2)	2104 (10.0)	138,334 (24.1)
EGTA + EGF	430 (4.1)	506 (2.4)	8,012 (1.40)
TPA (0.1 μg/ml)	607 (5.8)	916 (4.4)	17,220 (3.0)
EGTA + TPA	180 (1.7)	375 (1.79)	7,106 (1.23)
Insulin (1 μg/ml)	568 (5.4)	2612 (12.4)	62,566 (10.9)
Transferrin (10 μg/ml)	329 (3.1)	1454 (6.9)	26,978 (4.7)
Hydrocortisone (10^{-6} M)	74 (0.70)	142 (0.68)	8,610 (1.5)

Procedure 1, simultaneous application of growth factors and ^{32}P for 2 h; procedure 2, previous incorporation of ^{32}P into quiescent cells for 16 h, and application of growth factors for 20 min. [^{3}H]TdR was incorporated 5–20 h after stimulation.
* Ratio to the value without treatment.

DISCUSSION

These findings demonstrated that addition of mitogenic agents to intact quiescent 3Y1 cells rapidly enhanced Ca^{++}-dependent phosphorylation of 350,000- and 80,000-mol-wt proteins. The existence of two enzyme systems of Ca^{++}-dependent phosphorylation in the activation of platelets has been established; namely, Ca^{++}-activated phospholipid-dependent protein kinase (C-kinase) and calcium-calmodulin-dependent protein kinase (21). The crucial role of C-kinase in signal transduction for a variety of biologically active substances has also been shown (22). C-Kinase phosphorylates different amino acid residues from other kinases. A phorbol ester binds with C-kinase, which phosphorylates the EGF receptor, and reduces tyrosine protein kinase activity of the receptor (23, 24). We are now examining the involvement of C-kinase in the phosphorylations of the 350,000- and 80,000-mol-wt polypeptides in terms of phospholipid-dependency and the amino acid residues phosphorylated.

The 350,000- and 80,000-mol-wt proteins showed about 76% homology on two-dimensional peptide mapping (data not shown). This result suggests that cellular 350,000- and 80,000-mol-wt proteins belong to the same family with common antecedents. Although the map of MAP-1 of brain microtubules showed many peptide spots, the degradation products of MAP-1, 30,000- and 25,000-mol-wt polypeptides, exhibited substantial homology with cellular 350,000- and 80,000-mol-wt proteins (data not shown). Therefore, the cellular 350,000- and 80,000-mol-wt proteins are not identical with brain MAP-1, but are related molecules (manuscript submitted for publication).

The 350,000-mol-wt protein has been suggested to function as a linker between microtubules, intermediate filaments, and membrane proteins (6, 10, 11). In the present work 350,000- and 80,000-mol-wt polypeptides co-assembled with both microtubules and cold-resistant cytoskeletons. The immunofluorescent staining pattern of cytoplasmic network with anti-MAP-1 serum was that of the mixture of intermediate filaments and microtubules. Erythrocyte ankyrin, which links membrane protein to spectrin, is immunologically cross-reactive with brain MAP-1 and co-polymerizes with microtubules (25). Phosphorylation of these linker molecules may cause a decrease in their linking function and may result in transient dissociation of membrane molecules and cytoskeletal fibers. Their dissociation may function in transfer of a signal from the cell surface to the nucleus for induction of DNA synthesis. This speculation is consistent with the fact that agents disrupting microtubules induce DNA synthesis in quiescent cells (26, 27). Furthermore, addition of agents disrupting microtubules enhances the mitogenic activities of several growth factors, such as EGF, insulin, and phorbol esters (28, 29, 30). Insulin alone induces transient breakdown of microtubules, and EGF and insulin together induce perturbation of intermediate filaments (31). Centrosomal separation was also reported on mitogenic stimulation by EGF (9).

Phosphorylation of the 80,000-mol-wt protein has been detected 15 s after administration of phorbol esters, phospholipase C, or platelet-derived growth factor to quiescent 3T3 cells, though EGF and insulin did not have such rapid effects (32). Another interesting finding was that 80,000-mol-wt nonhistone nuclear protein is phosphorylated on transformation by Rous sarcoma virus (33). Studies are required on whether these 80,000-mol-wt phosphoproteins are identical with the 80,000-mol-wt protein immunoprecipitated by anti-MAP-1 antibody.

A monoclonal antibody against MAP-1 produced bright immunofluorescence on nuclear flecks and the cytoskeletal network and centrioles (7). The immunofluorescence of the nuclear flecks disappeared under growth-inhibiting conditions caused by serum starvation, and reappeared as the cells moved into the G_1 phase after addition of fresh serum (20). Thus it is possible that after stimulation the phosphorylations of 350,000- and 80,000-mol-wt polypeptides induce their release from cytoskeletons and their transfer to the nucleus. This might represent an early process of transduction of the signal of mitotic stimulation to the nucleus for induction of DNA synthesis. The co-localization of MAP-1 analogues and large T-antigen of SV40 and p53 on the nuclear skeleton has been observed (34).

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