A Cell Cycle *ts* Mutant, *ts*JT16, is Defective in p70 Synthesis Through Protein Kinase C-dependent and -Independent Pathways

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ABSTRACT. *ts*JT16 is a G₀/G₁ *ts* mutant from the Fischer rat fibroblast line. It has a *ts* defect in a function operating early after growth stimulation with fetal bovine serum (FBS). A primarily induced gene product, p70, was not synthesized at 40°C after stimulation with serum, while *c-fos* and *c-myc* mRNAs accumulated under the same condition. This paper reports that p70 was synthesized following stimulation of G₀-arrested cells with platelet-derived growth factor, epidermal growth factor (EGF), and 12-O-tetradecanoylphorbol-13-acetate (TPA) at 34°C, but not at 40°C. However, it was synthesized at both temperatures after addition of A23187. In protein kinase C-deprived cells, peptide growth factors and A23187 induced p70 at 34°C, whereas TPA did not. Fibroblast growth factor and insulin did not induce p70. Induction of *c-fos* and *c-myc* occurred at both temperatures after the stimulation with FBS, TPA or A23187. These results indicated that the defect in *ts*JT16 to induce p70 is likely to be located at the common downstream of protein kinase C-dependent and -independent pathways, but is independent from the pathway of calcium mobilization.

When G₀-arrested cells are stimulated to proliferate, polypeptide growth factors act by binding to specific cell surface receptors that transduce a broad range of signals leading to an induction of cell-cycle-dependent genes (1, 3, 5, 6). Although a number of low and high molecular weight functional molecules are known to participate in the signal transduction process, the molecular sequences or networks by which those growth signals are transduced into nuclei are yet far from fully understood. The use of an appropriate mutant defective in signal transduction processes would shed a new light on understanding this process.

We have reported a candidate for such a mutant, *ts*JT16, which was isolated from a rat fibroblast line (8, 9). G₀-arrested *ts*JT16 enters the S phase following stimulation with fetal bovine serum (FBS) at the permissive (34°C) temperature, but not at the nonpermissive (40°C) temperature. *ts*JT16 is a mutant defective in a function operating early, within 4 h, after growth stimulation with FBS, but accumulates mRNAs of such cell-cycle-dependent genes as *c-fos*, *c-myc* and ornithine decarboxylase at both 34°C and 40°C. However, *ts*JT16 is defective in inducing a nuclear labile protein, p70, that is synthesized very early after growth stimulation with FBS and disappears before the S phase. p70 is synthesized exclusively during G₀-S transition and not in the

growing cell cycle (10). The synthesis of p70 is transcriptionally controlled and is induced from primarily inducible genes. *ts*JT16 is expected to have a mutation in one of the signal transduction processes leading to induction of p70, but not in other processes leading to induction of *c-fos*, *c-myc* and ornithine decarboxylase genes. The purpose of the present report is to discover a possible difference in the signal transduction pathways operating after treatment with several types of growth stimuli.

MATERIALS AND METHODS

Materials. Materials used were: FBS from Whittaker Bio-products, Inc., Md.; human epidermal growth factor (EGF) from Wakunaga Pharmaceutical Co., Ltd., Hiroshima; platelet-derived growth factor (PDGF) from R & D Systems Inc.; fibroblast growth factor (FGF) from Toyobo Co., Ltd., Osaka; 12-O-tetradecanoylphorbol-13-acetate (TPA) from LC Service Corp.; A23187 and porcine insulin from Sigma Chemical Co.; and [methyl-³H]thymidine (74 GBq/m mol) from Amersham, Japan.

Cell culture and assay of DNA synthesis. *ts*JT16, a cell-cycle temperature-sensitive mutant from the Fischer rat fibroblast line, was cultured as previously described (8). The permissive temperature is 34°C and the nonpermissive temperature is 40°C. Subconfluent cells were growth arrested in the G₀ phase by culturing them in a serum-free medium for 2–3 days.

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Fig. 1. Induction of p70 after the stimulation of *G*₀-arrested *tsJT16* with various growth factors.

(A) *G*₀-arrested *tsJT16* was stimulated with 10% FBS (a, d), 25 ng/ml of EGF (b, e) or 10 ng/ml of PDGF (c, f) for 3 h at 34°C (a–c) or at 40°C (d–f) and labeled with [³⁵S]methionine for the last 2 h. *G*₀-arrested *tsJT16* was also labeled for 2 h at 34°C (g). (B) *G*₀-arrested *tsJT16* was stimulated with 10% FBS (a), 25 ng/ml of EGF, 50 ng/ml of FGF (c) or 10 µg/ml of insulin (d) for 3 h at 34°C and labeled with [³⁵S]methionine for the last 2 h. Whole cell proteins were analyzed by two-dimensional gel electrophoresis, followed by autoradiography. Arrow indicates p70.

They were stimulated to grow by changing the medium with that containing desired concentrations of mitogenic agents. DNA synthesis was assayed by measuring radioactivities incorporated into acid insoluble materials or by autoradiography after labeling cells with [³H]thymidine (74 KBq/ml) as described previously (9).

Detection of p70 synthesis and Northern blot analysis. Labeling of cells with [³⁵S]methionine and analysis of p70 on 2-dimensional gel electrophoresis was done according to the method reported previously (9, 10). Northern blot analysis of *c-fos* and *c-myc* was also carried out as previously described (9).

RESULTS

Induction of p70. p70 was induced in G₀-arrested *tsJT16* following stimulation at 34°C with FBS as reported previously (9). EGF and PDGF also induced p70 (Fig. 1A). Induction of p70 by these three stimulants was reduced when the cells were stimulated at 40°C. Although an additional protein spot with slightly higher molecular weight was also induced at 34°C after the stimulation with growth factors, but not at 40°C (Fig. 1), we tentatively referred to these two spots as p70 (9, 10). p70, was only a protein spot on 2-D gel whose synthesis was induced by growth factors and was temperature sensitive (9, 10). FGF and insulin did not induce p70 even at 34°C (Fig. 1B).

An activation of protein kinase C and an elevation of intracellular free Ca²⁺ ions was reported in many cell

types to mediate a transfer of the growth signal from the cell surface to the nuclei (1, 5, 6). Effects of the activator of protein kinase C, TPA (5), and the calcium ionophore, A23187, on induction of p70 were tested. Induction of p70 by TPA occurred and was temperature sensitive (Fig. 2). A23187 was found to induce p70 at both temperatures, rather extensively at 40°C (Fig. 2). These results suggest that the signal transduction pathway of the downstream of protein kinase C that leads to induction of p70 is inhibited at the nonpermissive temperature, whereas that of calcium mobilization is not.

However, this does not mean that FBS or other peptide growth factors induced p70 and DNA syntheses through a pathway mediated by activation of protein kinase C. *tsJT16* cells were growth arrested and deprived of protein kinase C by culturing confluent monolayer cells in a serum-free medium containing 100 nM of TPA for 48 h. TPA itself did not induce DNA synthesis (Fig. 3). Induction of DNA synthesis occurred equally in both protein kinase C-deprived and undeprived control cells after serum stimulation (Fig. 3). Similar results were obtained in 3Y1 which is a parental line of *tsJT16*. Staurosporine, an inhibitor of protein kinase C, did not at all inhibit an induction of DNA synthesis in FBS-stimulated *tsJT16* cells (data not shown). After the stimulation of G₀-arrested cells with PDGF, EGF or insulin, DNA synthesis was induced and was partly inhibited by the deprivation of protein kinase C (Fig. 3A). These phenomena indicated that protein kinase C was not required for cells entering into the S phase fol-

Fig. 2. Induction of p70 after the stimulation of G₀-arrested *tsJT16* with TPA and A23187.

G₀-arrested *tsJT16* was stimulated with 10% FBS (a, d), 100 nM of TPA (b, e) or 100 nM of A23187 (c, f) for 3 h at 34°C (a-c) or at 40°C (d-f) and labeled with [³⁵S]methionine for the last 2 h. Whole cell-proteins were analyzed by two-dimensional gel electrophoresis. Arrow indicates p70.

Fig. 3. Induction DNA synthesis in protein kinase C-deprived *tsJT16*.

G₀-arrested (open column) or protein kinase C-deprived and G₀-arrested (hatched column) *tsJT16* was stimulated with 10% FBS (b), 10 ng/ml of PDGF (c), 10 ng/ml of EGF (d), 100 nM of TPA (e), 100 nM of A23187 (f), 50 ng/ml FGF (g) or 10 $\mu\text{g/ml}$ of insulin (h). The cells were cultured in the presence of [^3H]thymidine at 34°C for 36 h and the radioactivities incorporated into acid insoluble material were measured. Unstimulated control was also assayed (a).

lowing serum stimulation, and was required only partly following stimulation with PDGF, EGF or insulin. When the protein kinase C-deprived cells were stimulated with TPA, they did not synthesize p70 at all (Fig. 4). Induction of p70 by FBS, PDGF or EGF did not, or did partly, reduced in cells deprived of protein kinase C. Induction of p70 by A23187 was not at all reduced by deprivation of protein kinase C.

These results indicate that: 1) the protein kinase C-dependent pathway that is activated by TPA is temperature sensitive to induce p70; 2) serum, PDGF or EGF induces p70 through both pathways, dependent and independent, of protein kinase C, although the latter is the major pathway; 3) all these pathways are temperature sensitive; and 4) A23187 activates the protein kinase C-independent pathway which is not temperature sensitive.

Induction of *c-fos* and *c-myc*. Induction of *c-fos* and *c-myc* genes after FBS stimulation of G₀-arrested *tsJT16* was monitored by Northern blot analysis and found to be independent of culture temperature (9). Although the extent of the expression of these genes va-

ried after stimulation with different stimulators such as TPA and A23187, it was no lower at 40°C than at 34°C (Fig. 5). An induction of *c-fos* and *c-myc* by FBS or A23187 was not blocked by deprivation of protein kinase C, but that by TPA was in 3Y1 cells (unpublished data), facts that are inconsistent with the above results that the downstream of FBS or A23187 stimulation was independent from the activation of protein kinase C in *tsJT16*.

Inability of entrance into the S phase at 40°C in the presence of A23187 and serum. An induction of DNA synthesis after FBS stimulation of G₀-arrested *tsJT16* appeared to correlate to that of p70, but not to that of *c-fos* and *c-myc*. Since A23187 could induce p70 at both permissive and nonpermissive temperatures, induction of DNA synthesis at 40°C was expected after treatment of G₀-arrested *tsJT16* with A23187 or A23187 plus FBS. Although A23187 was used at 100 nM for the short-term experiments described above without any apparent cytotoxic effect, it was cytotoxic for long-term experiments to see the induction of DNA synthesis. For long-term experiments, longer than 1 day, 10 nM was the highest limit of concentration that avoided the cytotoxic effect. A23187 at 10 nM could induce a significant amount of p70 protein and *c-myc* mRNA, although the extent of this induction was smaller than that at 100 nM (data not shown). As shown in Table I, however, when the cells were stimulated at 40°C with 10% FBS plus 10 nM of A23187, DNA synthesis did not occur, indicating that an activation of these cell-cycle-dependent genes as *c-fos*, *c-myc*, and p70 was not sufficient for an induction of DNA synthesis in *tsJT16* cells, although this did not negate the premise that activation of these genes is required for induction of DNA synthesis.

DISCUSSION

Since cDNA for p70 has not yet been available, we could not directly study the induction of p70 genes at the mRNA level. However, previous studies suggested that: 1) the synthesis of p70 is transcriptionally controlled, 2) the p70 gene is primarily induced, 3) mRNA of p70 is synthesized exclusively within 2 h after growth stimulation at 34°C of G₀-arrested *tsJT16* cells, and 4) the function mutated in *tsJT16* is concerned with the process to activate p70 genes (9, 10). These biochemical results were compatible to the cell cycle studies that purported that *tsJT16* can traverse toward the S phase at 40°C, following the 34°C incubation for the first 3–4 h after growth stimulation (8).

Increasing information on the process of signal transduction revealed complicated signal transfer network from the cell membrane to the nuclei (1, 5, 6). This network is qualitatively different among various cell types, and it functions in quantitatively different ways, re-

Fig. 4. Induction of p70 in protein kinase C-deprived *tsJT16*.

(A) G₀-arrested (a–c) or protein kinase C-deprived and G₀-arrested (d–f) *tsJT16* (d–f) was stimulated with 10% serum (a, d), 100 nM of TPA (b, e) or 100 nM of A23187 (c, f) for 3 h at 34°C and labeled with [³⁵S]methionine for the last 2 h. (B) G₀-arrested (a, b) or protein kinase C-deprived and G₀-arrested (c, d) *tsJT16* was stimulated with 10 ng/ml of EGF (a, c) or 10 ng/ml of PDGF (b, d) for 3 h at 34°C and labeled with [³⁵S]methionine for the last 2 h. Whole cell proteins were analyzed by two-dimensional gel electrophoresis. Arrow indicates p70.

sponding to the various growth factors found even in the same cell type. Several peptide growth factors activate the turnover of membrane phosphatidylinositol, leading to the activation of protein kinase C and to the elevation of the cytoplasmic concentration of free Ca²⁺. In *tsJT16* cells, an induction of p70 occurred by TPA, a potent activator of protein kinase C (5). Although it has been known that protein kinase C is activated by several peptide growth factors, the major pathway for the induction of p70 by FBS, PDGF, and EGF

was not mediated by protein kinase C in *tsJT16*. This was consistent with the observations that 3Y1, a parental line of *tsJT16*, showed very weak, if any, activity of protein kinase C (unpublished data). The downstream of both protein kinase C-dependent and -independent processes was temperature sensitive in inducing p70. *c-fos* and *c-myc* were induced by FBS not through protein kinase C-activation, or by TPA through protein kinase C activation, and neither of these processes were temperature sensitive. Therefore, each process that is mediated

Fig. 5. Induction of *c-fos* and *c-myc* after the stimulation of *tsJT16* with various growth factors.

G_0 -arrested *tsJT16* was stimulated with 100 nM of TPA, 100 nM of A23187 or 10% serum for 30 min for *c-fos* expression (a) or for 4 h for *c-myc* expression (b). Cytoplasmic RNA (20 μ g/lane) was analyzed by Northern blot analysis. p; stimulated at the permissive temperature (34°C), np; stimulated at the nonpermissive temperature (40°C), US; unstimulated control.

or not mediated by protein kinase C is thus differentiated further into temperature-sensitive (for p70 induction) and -insensitive (for *c-fos* and *c-myc* induction) pathways.

PDGF, EGF and FBS are also known to induce calcium mobilization (2, 4, 7, 12). Although the downstream of calcium influx by A23187 is as yet unclear in *tsJT16*, the resulting induction of p70, *c-fos* and *c-myc* was not temperature-sensitive. Could the upstream process of calcium mobilization after stimulation with peptide growth factors be yet another candidate of the temperature-sensitive event in *tsJT16* cells? Changes in intracellular free calcium levels had to be measured after treatment of *tsJT16* with peptide growth factors at both 34 and 40°C, but could not be performed because of the limitations of temperature-controlled equipment. Although we do not have a direct answer at present, it may be reasonable to assume that even when the increase in a concentration of cytoplasmic free Ca^{2+} occurred after the stimulation with peptide growth fac-

Table I. INABILITY OF *tsJT16* TO ENTER S PHASE AT THE NON-PERMISSIVE TEMPERATURE AFTER TREATMENT WITH SERUM AND A23187.

	Culture temperature (°C)	labeled nuclei (%)
US	34	15.5
FBS	34	79.8
	40	13.4
FBS + A23187	40	17.7

G_0 -arrested *tsJT16* was unstimulated (US) or stimulated with 10% FBS or 10% FBS plus 10 nM A23187, cultured at the permissive (34°C) or nonpermissive (40°C) temperatures for 48 h in the presence of 74 MBq/ml of [3H]thymidine, and processed for autoradiography.

tors, it may be lower than that artificially induced by A23187 treatment and may not be enough by itself to activate p70 genes at 40°C. In contrast with peptide growth factors, however, TPA was not known to activate calcium mobilization, and therefore the reason why TPA failed to induce p70 at 40°C could not be the failure of the calcium mobilization at 40°C. In *tsJT16* cells, FBS, PDGF and EGF appeared to induce p70 mainly through a temperature-sensitive pathway which is independent from an activation of protein kinase C and Ca^{2+} mobilization. The presence of such a pathway that is independent from an activation of protein kinase C and Ca^{2+} mobilization was also reported in 3T3 cells, which resulted in an induction of nuclear genes following stimulation with EGF (11).

Cell-cycle-dependent genes are activated through the functions of both *cis*- and *trans*-elements, and are controlled by multiple *cis*- and *trans*- elements (3). One of the simple models for the induction of p70 is that the *cis*-element of the p70 gene is composed of at least two parts: one is responsible for serum, PDGF, EGF or

Fig. 6. Possible signal transduction pathways in *tsJT16*.

TPA and the other is responsible for calcium mobilization (Fig. 6). The former *cis*-element(s) is activated by a *trans*-element(s) whose activation is mediated by either a protein kinase C-dependent or -independent pathway and is temperature sensitive. The latter *cis*-element responsible for the extremely high concentration of free calcium is activated through the temperature-insensitive pathway which is independent from the stimulation with serum, PDGF, EGF, or TPA. Studies on *trans*-element factors from cells stimulated with various agents at either the permissive or nonpermissive temperature should be analyzed to validate this model and to clarify the temperature-sensitive element in *tsJT16*.

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