Volume 226, number 1, 53–57 FEB 05404 December 1987

A 63 kDa protein is secreted from BALB/c-3T3 cells entering the G_1 phase from the G_0 state

Shin-ichi Tominaga

Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato-ku, Tokyo 105, Japan

Received 2 November 1987

A 63 kDa protein is detectable in the culture fluid of mouse BALB/c-3T3 cells traversing from the G_0 state to the G_1 phase, whereas it is undetectable in the culture fluid of quiescent or growing BALB/c-3T3 cells. Secretion of the protein is maximal at 10 h after serum addition. G_0-specific ts mutant cells (rat tsJT60) also secrete the 63 kDa protein only when the quiescent cells are stimulated by serum addition at permissive temperature. These facts indicate that the 63 kDa protein is secreted only from cells traversing from the G_0 state to the G_1 phase.

Cell cycle; Cell growth; Protein secretion; Interferon

1. INTRODUCTION

The transition from the G_0 state to the G_1 and S phases is a key step in cell proliferation [1]. The levels of several mRNAs (including those for c-myc, c-fos and JE) increase transiently during this transition [2–4]. Tominaga and Lengyel [5] reported that the exposure of quiescent BALB/c-3T3 cells to PDGF (which results in the entry of cells into the G_1 and S phases) also resulted in the secretion of a 63 kDa protein [5]. Furthermore, when the cells were pretreated with 1000 IU/ml of purified mouse β-IFN [6] for 48 h prior to exposure to PDGF, the 63 kDa protein was scarcely detectable in the culture fluid of PDGF-stimulated cells. The treatment with IFN also decreased the percentage of cells undergoing cell division after exposure to PDGF (in platelet-poor plasma). Here, I present results of experiments with two cell lines (mouse BALB/c-3T3 and rat tsJT60). The data indicate that the 63 kDa protein is secreted only from cells traversing from the G_0 state to the G_1 phase.

Correspondence address: S. Tominaga, Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato-ku, Tokyo 105, Japan

Abbreviations: IFN, interferon; DME, Dulbecco's modified Eagle's medium; MEM, minimum Eagle's medium; PDGF, platelet-derived growth factor

2. MATERIALS AND METHODS

BALB/c-3T3 cells (clone A31) were provided by Dr C. Stiles (Harvard Medical School), ts Ki ras transformed BALB/c-3T3 cells (tsKi-3T3-714) were a kind gift from Dr B. Peterkofsky (National Cancer Institute) [7] and tsJT60 cells were from Dr T. Ide (Hiroshima University, Japan) [8]. DME, MEM, calf serum and fetal bovine serum were from Gibco. MEM (– methionine) was from Nissui Pharmaceutical. [^{35}S]Methionine (spec. act. 1130 Ci/mmol) was from Amersham.

Cell number was counted under a phase-contrast microscopy using a hematocytometer [5]. The average of triplicate counts was taken as the cell number. Cells were cultured and labeled with 40 μCi/ml of [^{35}S]methionine for 1 h, and the culture fluid fractionated by gel electrophoresis in a 10–20% linear gradient polyacrylamide gel in the presence of SDS and visualized by fluorography as

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

in [5]. A [^{14}C]methylated protein mixture from Amersham was used as size marker. Densitometry of the fluorogram was carried out using a Shimadzu dual-wavelength TLC scanner and C-R1A chromatopac.

3. RESULTS AND DISCUSSION

The results in fig.1 reveal that the 63 kDa protein was undetectable in the culture fluid of quiescent BALB/c-3T3 cells (lane c). The protein was also undetectable in the culture fluid of growing cells at two different cell densities, i.e. 30 and 70% (fig.1, lanes a,b). The 63 kDa protein was detectable only when quiescent cells were stimulated by serum addition. Secretion was maximal at 10 h, just before the initiation of DNA synthesis which occurs at 12 h in this cell line [9,10]. Thereafter, the secretion decreased gradually (see table 1). No second peak of secretion of this protein was noted. In addition, a PDGF-induced 32 kDa secreted protein [5] was also maximally detectable at 10 h (arrowhead in fig.1). Thus, both the 63 and 32 kDa proteins were maximally secreted before the BALB/c-3T3 cells entered into the S phase from the G_0 state.

The search for the secretion of the 63 kDa protein in the culture fluids of various transformed or

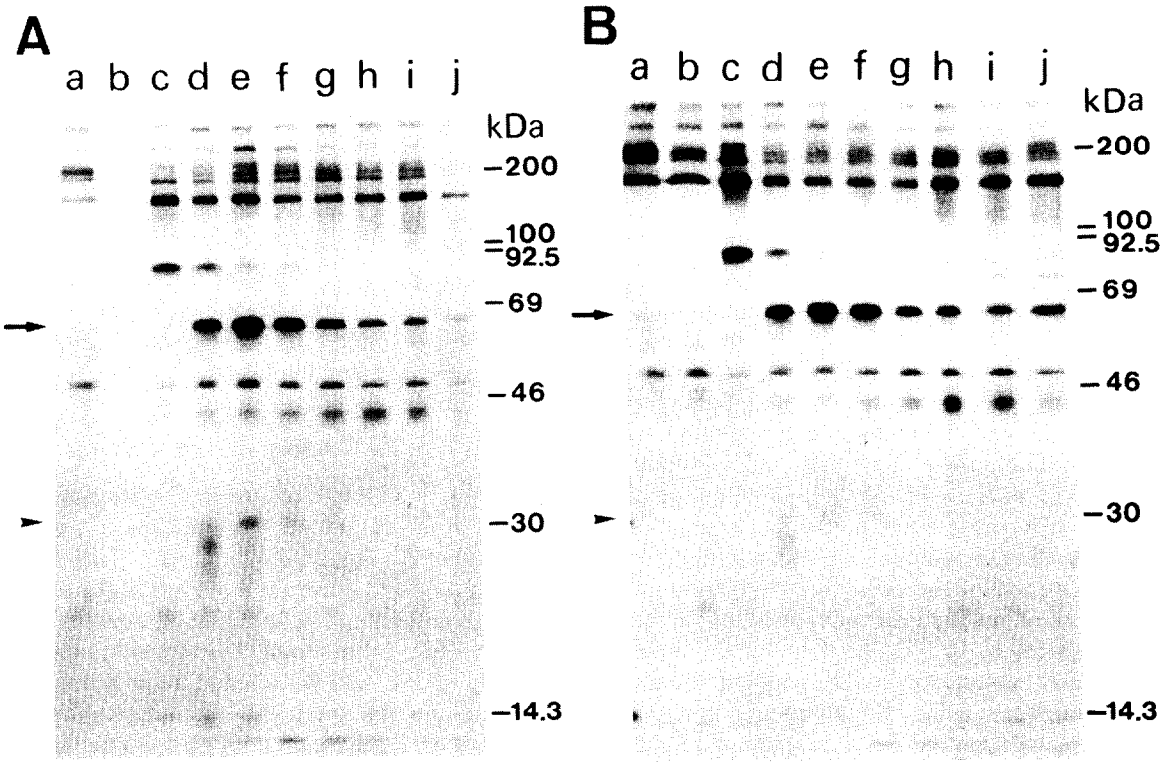

Fig.1. Time course of secretion of the 63 kDa protein from BALB/c-3T3 cells. Growing BALB/c-3T3 cells (lanes a,b) (1 and 2 days after a split in a 1:5 ratio in DME + 10% calf serum, having reached 30 and 70% confluency, respectively) were incubated in the presence of [^{35}S]methionine for 1 h as described [11]. Quiescent BALB/c-3T3 cells in DME + 5% platelet-poor plasma [5,11] were washed and incubated with DME + 10% calf serum for 5 (lane d), 10 (lane e), 15 (lane f), 20 (lane g), 30 (lane h), 40 (lane i), and 50 h (lane j), and labeled with [^{35}S]methionine for 1 h. Unstimulated quiescent BALB/c-3T3 cells in DME + 5% platelet poor plasma were labeled for 1 h at 10 h (lane c). (A) Cell numbers were counted after harvesting the culture fluid, and an amount of culture fluid corresponding to 1×10^5 cells were fractionated by gel electrophoresis and visualized by fluorography as described in section 2. (B) An amount corresponding to 20000 trichloroacetic acid-precipitable cpm of the culture fluid was analyzed as in (A). The arrow indicates the position of the 63 kDa protein. The arrowhead denotes the position of a 32 kDa protein.

Table 1

Variation in intensity of the 63 kDa protein band with time elapsed after the addition of serum to the quiescent cells

Time elapsed (h)	Intensity (%)
2	0
4	33.98 ± 4.29
6	40.10 ± 3.60
8	70.50 ± 11.3
10	100
12	76.25 ± 3.05
16	44.95 ± 4.92
20	18.08 ± 6.31

Four experiments of 1 h labeling from 2 to 20 h after serum stimulation were performed, and 20000 trichloroacetic acid-precipitable cpm was fractionated by gel electrophoresis, the fluorogram being processed for densitometry as described in section 2. In all four experiments, the maximum densities were at 10 h, and the value at 10 h was taken as 100% in each case. Values were calculated and are means ± SD

cancer cells revealed that only one Kirsten ras transformed NIH 3T3 cell line (K-NIH) excreted the protein continuously [11]. The question was whether the appearance was specific for Ki ras transformation. Ts Ki ras transformed BALB/c-3T3 cells (tsKi-3T3-714) were available for studying this problem [7]. As shown in fig.2, contrary to my expectation, the 63 kDa protein was detectable at the non-permissive temperature (40°C), at which the cells showed the normal phenotype of cobblestone appearance. The identity of the band was suggested by the observation that treatment with purified IFN decreased it by 50% [11]. On the other hand, at the permissive temperature (36°C), the 63 kDa protein was not detectable, suggesting that the appearance of this protein was not universal in Ki ras transformed cells. One of the possible explanations is that the 63 kDa protein is secreted only at G_0/G_i transition. Ts mutant cells of a cancerous phenotype, such as tsKi-3T3-714 cells, enter the G_0 state only at non-permissive temperature [12,13]. Some of the cells at non-permissive temperature may enter the G_0 state and a proportion of these cells re-enter the G_1 phase, since the cells may secrete some growth factors in an autocrine fashion. Therefore, the

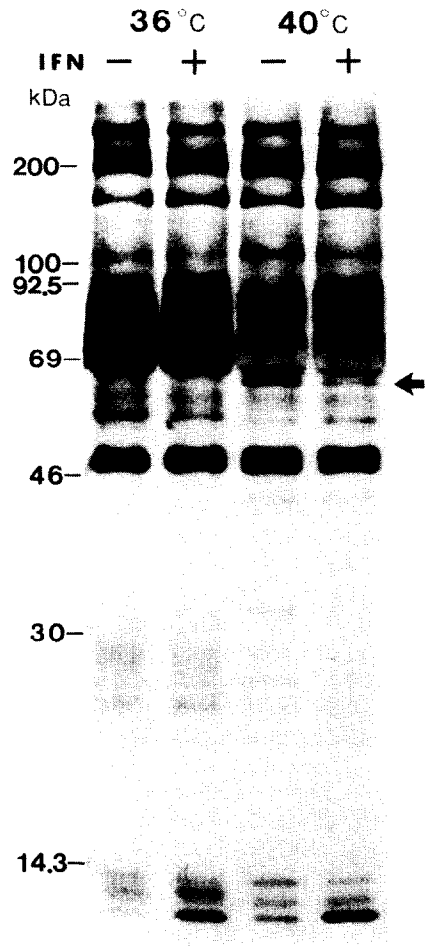

Fig.2. Appearance of the 63 kDa protein in the culture fluid of tsKi-3T3-714 cells at non-permissive temperature. TsKi-3T3-714 cells were split at a 1:5 ratio in MEM + 10% fetal bovine serum, kept at 36°C for 48 h. Cells were supplemented with 1000 IU/ml of purified mouse β-IFN (5×10^8 IU/mg) [6], where indicated, and kept at either 36°C (2 lanes on left) or 40°C (2 lanes on right) for 48 h. Cells were then labeled with [^{35}S]methionine for 1 h. An amount corresponding to 10000 trichloroacetic acid-precipitable cpm of the culture fluid was analyzed as in fig.1. The arrow indicates the position of the 63 kDa protein.

63 kDa protein could be detected in the culture fluid of tsKi-3T3-714 cells at non-permissive temperature.

This working hypothesis prompted me to use a G_0-specific ts mutant, tsJT60 cells. These cells were derived from rat 3Y1 cells [8]. As shown in fig.3A,

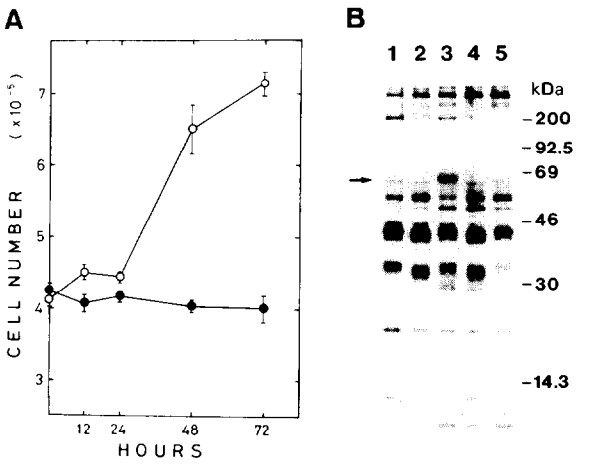

Fig.3. Appearance of the 63 kDa protein in culture fluid of tsJT60 cells at the permissive temperature. (A) Quiescent tsJT60 cells were serum stimulated as described in (B) at either 34°C (○—○) or 39.5°C (●—●). Cell number was counted at the indicated times. (B) TsJT60 cells were split in a 1:5 ratio in DME + 10% fetal bovine serum and cultured at 34°C for 5 days to obtain confluent cells. Quiescent cells were washed, supplemented with DME + 5% platelet-poor plasma and cultured at 34°C for 24 h. Cells were then transferred to a 39.5°C chamber at 7% CO_2 (lanes 2,4) or kept at 34°C at 5% CO_2 (lanes 1,3), and cultured for a further 24 h. Cells were washed and supplemented with DME + 10% fetal bovine serum (lanes 3,4) or remained in DME + 5% platelet-poor plasma (lanes 1,2). Cells were washed 6 h after serum addition and labeled with [^{35}S]methionine for 1 h. Growing cells were obtained 2 days after splitting in a 1:5 ratio, having reached 50% confluency, and labeled with [^{35}S]methionine for 1 h (lane 5). An amount corresponding to 20000 trichloroacetic acid-precipitable cpm of the culture fluid was fractionated and analyzed as described in fig.1. The arrow indicates the position of the 63 kDa protein.

tsJT60 cells maintained in the G_0 state and stimulated by serum addition entered into the cell cycle at permissive temperature (34°C), but not at non-permissive temperature (39.5°C). However, treatment at 39.5°C did not affect the growth of the continuously dividing cells [8] (not shown), suggesting that the ts mutation was not in the G_1 phase but at the G_0/G_1 transition. After confirming these conditions, the experiment shown in fig.3B was performed. The 63 kDa secreted protein was detected only in the culture fluid of serum-stimulated quiescent tsJT60 cells at the permissive temperature (34°C) (fig.3, lane 3), being undetectable at the non-permissive temperature (39.5°C) (fig.3, lane 4). The 63 kDa protein was also undetectable in the culture fluid of non-stimulated quiescent tsJT60 cells at both permissive (fig.3, lane 1) and non-permissive (fig.3, lane 2) temperatures, and in the culture fluid of growing tsJT60 cells (fig.3, lane 5). (A band of slightly faster mobility could be seen in all lanes of fig.3B, but this band was distinct from the 63 kDa protein in repeated experiments.) These results reveal that tsJT60 cells resemble BALB/c-3T3 cells in secreting a 63 kDa protein when traversing from the G_0 state to the G_1 phase. Since purified rat IFN was not available to me and the sensitivity to IFN of rat 3Y1 cells is not known, I could not investigate the effect of IFN on the secretion of the 63 kDa protein from tsJT60 cells. Although the elucidation of the biological function(s) of this 63 kDa protein must await further study, its secretion may serve as a marker for cells traversing from the G_0 state to the G_1 phase.

ACKNOWLEDGEMENTS

I thank Dr Beverly Peterkofsky for providing tsKi-3T3-714 cells, Dr Toshinori Ide for tsJT60 cells, and Dr Peter Lengyel for helpful discussions and reading the manuscript. This work was supported in part by a research grant from the Japanese Ministry of Education, Science and Culture.

REFERENCES

[1] Pardee, A.B., Dubrow, R., Hamlin, J.L. and Kletzien, R.F. (1978) Annu. Rev. Biochem. 47, 715–750.
[2] Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983) Cell 35, 603–610.
[3] Greenberg, M.E. and Ziff, E.B. (1984) Nature 311, 433–438.
[4] Cochran, B.H., Reffel, A.C. and Stiles, C.D. (1983) Cell 33, 939–947.
[5] Tominaga, S. and Lengyel, P. (1985) J. Biol. Chem. 260, 1975–1978.

[6] Jayaram, B.M., Schmidt, H., Yoshie, O. and Lengyel, P. (1983) J. Interferon Res. 3, 177–180.
[7] Bateman, J.F. and Peterkofsky, B. (1981) Proc. Natl. Acad. Sci. USA 78, 6028–6032.
[8] Ide, T., Ninomiya, J. and Ishibashi, S. (1984) Exp. Cell Res. 150, 60–67.
[9] Sokawa, Y., Watanabe, Y., Watanabe, Y. and Kawade, Y. (1977) Nature 268, 236–238.
[10] Balkwill, F. and Taylor-Papadimitriou, J. (1978) Nature 274, 798–800.
[11] Tominaga, S., Tominaga, K. and Lengyel, P. (1985) J. Biol. Chem. 260, 16406–16410.
[12] Renger, H.C. and Basilico, C. (1972) Proc. Natl. Acad. Sci. USA 69, 109–114.
[13] Yamaguchi, N. and Weinstein, B. (1975) Proc. Natl. Acad. Sci. USA 72, 214–218.