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Phosphorylation of the ETS-2 Protein: Regulation by the T-Cell Antigen Receptor-CD3 Complex

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Phosphorylation of the human *ets-2* protein in response to mitogenic signals to T lymphocytes was investigated in Jurkat cells. Activation of the cells by antibodies against the T-cell antigen receptor-CD3 complex or by concanavalin A was followed within 5 min by increased phosphorylation of the protein, as shown by a mobility shift of the protein from 54 to 56 kilodaltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and increased incorporation of ^{32}P. The Ca^{2+} ionophores A23187 and ionomycin were able to mimic this effect, suggesting that this phosphorylation is mediated by Ca^{2+}.

The retroviral oncogenes were derived from cellular genes, termed proto-oncogenes, which are normally involved in the signaling systems regulating various cellular functions, including proliferation and differentiation (23). The avian retrovirus E26 contains two cell-derived sequences, v-*myb*^{E} and v-*ets*, and both of them are involved in cellular transformation by the virus (9, 11–13, 25). The cellular homologs of the v-*ets* sequence constitute the *ets* gene family which includes *ets-1*, *ets-2*, *erg*, *elk-1*, and *elk-2* (4, 16–18, 22). The functional importance of the gene family is suggested by the fact that it is highly conserved in a wide range of phylogeny, from drosophila to humans (5, 15, 21). Nuclear proteins of about 60 kilodaltons were previously identified as the products of the human and chicken *ets-2* genes (4, 8). The *ets-2* proteins are similar to other nuclear proto-oncogene products with respect to quick turnover, phosphorylation, and modulation of expression by external stimuli (2, 4, 7).

In our attempt to find a possible association between the *ets-2* protein and the cellular signaling systems, we have been characterizing the posttranslational modification of *ets-2* protein in response to the intracellular second messengers. Ca^{2+} and protein kinase C are particularly interesting because they have essential roles in the regulation of T-lymphocyte functions (10, 20, 24), and high expression of the *ets-2* protein in the thymus suggests that it has a role in these T-cell regulatory processes (2, 3). We have previously reported that activation of protein kinase C stabilizes the labile *ets-2* protein (7). Here we present evidence that the phosphorylation of the *ets-2* protein in a human T-cell line is stimulated by mitogenic signals mediated by a Ca^{2+}-mediated mechanism.

We chose the human mature T-cell line Jurkat for our investigation because this cell line has been well characterized as a model system to study early signaling mechanisms in T-cell activation. Jurkat cells were labeled with [^{35}S] methionine or $^{32}P_i$, and the cellular lysate was immunoprecipitated by *ets-2*-specific polyclonal or monoclonal antibodies raised against a bacterially expressed human *ets-2* protein (7, 8). The results (Fig. 1A) indicated that the *ets-2* protein isolated from [^{35}S]methionine-labeled Jurkat cells consisted of at least two components, a diffuse 56-kilodalton band (p56) and a sharp 54-kilodalton band (p54), as fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These two components were competed out by the bacterially expressed human *ets-2* protein which was used as an antigen to prepare the antibodies (Fig. 1A, lanes 1 and 3). Peptide mapping with staphylococcal V8 protease gave almost identical peptide profiles for p56 and p54 except for a few additional fragments from p54 (Fig. 1B). p56 has a peptide map identical to that of the 56-kilodalton *ets-2* protein originally identified in the human Colo 320 DM and CEM cells, in which the p54 was not clearly detected by the polyclonal antibody (7, 8) (data not shown). From the $^{32}P_i$-labeled cells, both the polyclonal and monoclonal antibodies detected only p56 (Fig. 1A, lanes 5 and 7). These data indicate that both p56 and p54 are products of the *ets-2* gene and suggest that p56 is a phosphorylated form of p54. The minor difference in the peptide maps of p56 and p54 could be the result of phosphorylation, or it may reflect the possible heterogeneity of p54. Treatment of the [^{35}S]methionine-labeled immunoprecipitate with acid phosphatase converted p56 to p54 in a dose-dependent manner, supporting the conclusion that p56 is the phosphorylated form of p54 (Fig. 2C). Acid phosphatase treatment, however, did not eliminate p56 completely, leaving the possibility that p56 is heterogeneous and contains not only the phosphorylated form of p54 but some additional component of the *ets-2* protein (Fig. 2C). Since the polyclonal and monoclonal antibodies gave the same results in immunoprecipitation, we used the monoclonal antibody in the following experiments.

Antibodies with mitogenic activity directed against the T-cell receptor-CD3 complex or T-cell-specific mitogens such as concanavalin A increase the cytoplasmic concentration of Ca^{2+} which, together with protein kinase C, plays an essential role in mitogenic activation of T lymphocytes (10, 20, 24). To investigate the possible effect of these mitogenic stimuli on the phosphorylation of *ets-2* protein, Jurkat cells prelabeled with [^{35}S]methionine were treated briefly (10 min) with the antibodies against the various T-cell surface markers or with mitogens and the *ets-2* protein was examined by immunoprecipitation. The results shown in Fig. 3A indicate that the antibodies against the T-cell antigen receptor (Becton Dickinson and Co., Paramus, N.J.) or the OKT3 marker (identical to CD3) as well as the lectin mitogen concanavalin A induced a mobility shift of the *ets-2* protein in SDS-

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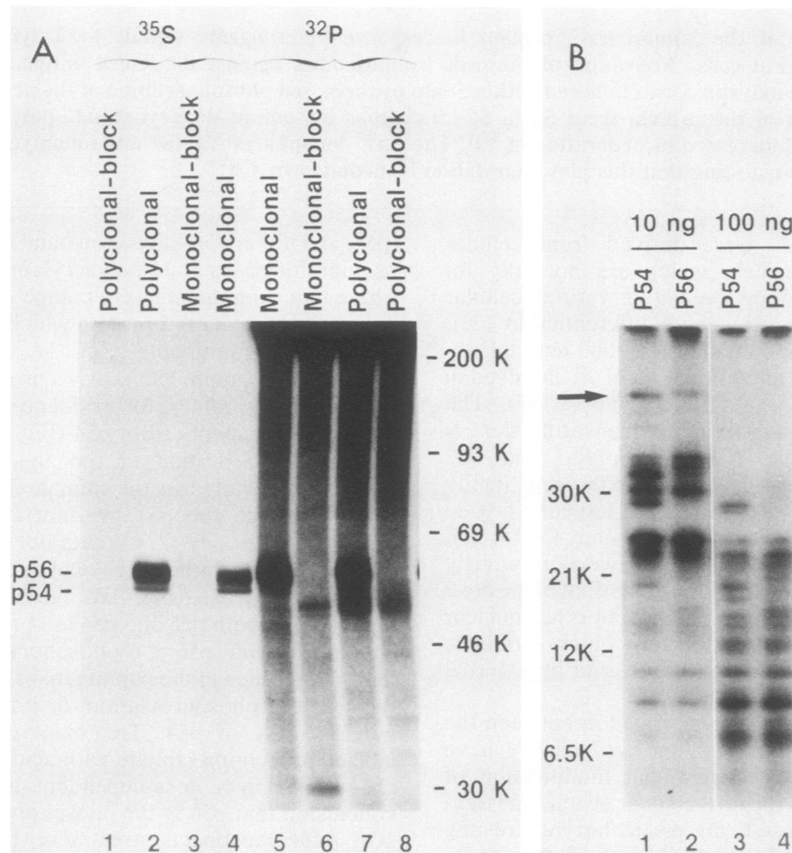

FIG. 1. Human *ets-2* proteins immunoprecipitated from Jurkat cells. (A) Cells were labeled with [^{35}S]methionine (lanes 1 through 4) or $^{32}P_i$ (lanes 5 through 8), and the cell lysates were immunoprecipitated with the *ets-2*-specific polyclonal (lanes 1, 2, 7, and 8) or monoclonal (lanes 3, 4, 5, and 6) antibody. Competition with the immunogen is shown in lanes 1, 3, 6, and 8. The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis (9% acrylamide) and visualized by fluorography. Relative mobilities of the standard proteins are shown on the right side of the panel (K, $\times 10^3$). The two *ets-2* components p56 and p54 are indicated. Five times more cells were used for labeling with ^{32}P than for ^{35}S labeling. (B) Staphylococcal V8 protease mapping of the *ets-2* proteins. Portions of the SDS-polyacrylamide gel containing p56 or p54 were cut out and processed by the method described by Cleveland and others (6). Samples of 10 ng (lanes 1 and 2) or 100 ng (lanes 3 and 4) of the enzyme were added to the lane. The intact p56 and p54 are indicated by an arrow.

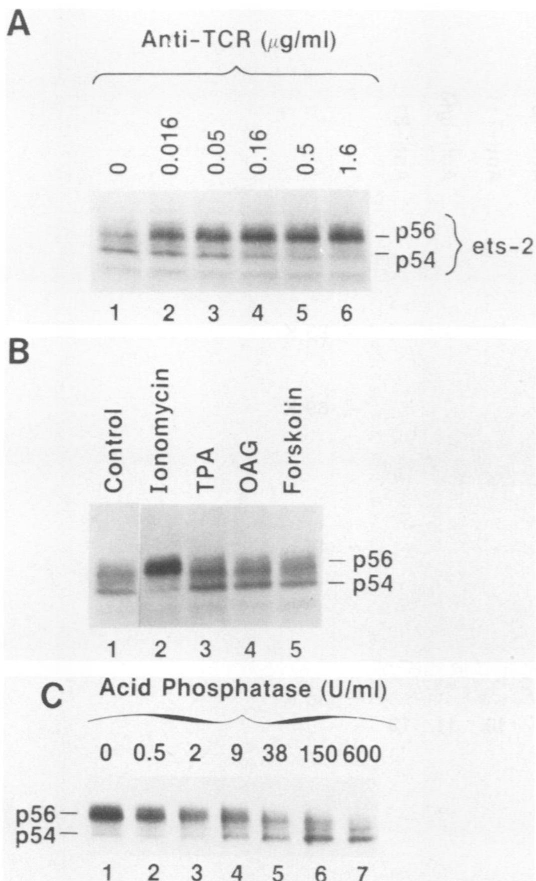

FIG. 2. Characterization of *ets-2* protein phosphorylation. (A) Dose response to the antibody against the T-cell antigen receptor (TCR). After being prelabeled for 3 h, Jurkat cells were treated with various concentrations of the antibody as indicated and the *ets-2* protein was detected by immunoprecipitation. (B) Prelabeled Jurkat cells were incubated for 10 min with ionomycin (0.5 μM) (lane 2), TPA (10 nM) (lane 3), OAG (100 μM) (lane 4), or Forskolin (30 μM) (lane 5), and the *ets-2* protein was detected by immunoprecipitation. A sample of the prelabeled cells remained untreated as a control (lane 1). (C) Treatment with acid phosphatase. The *ets-2* protein was immunoprecipitated from Jurkat cells after treatment with antibody to T-cell antigen receptor (1 μg/ml) and incubated (37°C, 30 min) with various concentrations of potato acid phosphatase as indicated.

polyacrylamide gels; in the unstimulated cells, p54 was dominant relative to p56; however, after stimulation, p56 became prominent and p54 was almost invisible, suggesting that phosphorylation of the protein was stimulated. This mobility shift should involve only posttranslational modification, because the protein synthesis inhibitor cycloheximide did not affect it (data not shown). Antibodies against other T-cell surface markers, including OKT4, OKT8, and OKT11, or B-cell-specific B1 marker and immunoglobulin M did not have this effect. This mobility shift was shown to depend on the concentration of the antibody added (Fig. 2A). Increased incorporation of $^{32}P_i$ into the p56 component was also shown after stimulation by the antibody against the antigen receptor (Fig. 3B). These results indicate that mitogenic stimuli to the T-cell antigen receptor-CD3 complex examined above induce phosphorylation of the *ets-2* protein and that this response is probably mediated by Ca^{2+}, because the Ca^{2+} ionophores A23187 (Fig. 3A, lane 5, and Fig. 3B, lane 5) and ionomycin (Fig. 2B, lane 2) also induced the same effect. The results of our unpublished experiments showed that *ets-2* protein phosphorylation is fully stimulated within 5 min after the addition of the antibody against the antigen receptor. The protein kinase C activators 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetyl-glycerol (OAG) and the adenylate cyclase agonist Forskolin did not induce this mobility shift of the *ets-2* protein, suggesting that protein kinase C and the cyclic AMP-dependent protein kinase are not the major factors in the regulation of the *ets-2* protein phosphorylation in Jurkat cells (Fig. 2B). Phosphoamino acid analysis of the *ets-2* protein immunoprecipitated from Jurkat cells treated with antibody against the T-cell antigen receptor showed that the protein is mainly phosphorylated at the serine residue(s) with a trace amount of phosphothreonine, which was visible only after overexposure of the X-ray film (Fig. 3C). Since interleukin-2 production by Jurkat cells requires the synergistic action of the Ca^{2+}- and protein kinase C-mediated signals (24), we treated the prelabeled Jurkat cells with a combination of TPA and the antibody to the antigen receptor and examined the phosphorylation of the *ets-2* protein. The results showed that the increase in the phosphorylation level of the protein was less marked than that seen after stimulation by the antibody to the antigen receptor alone (data not shown). This is consistent with the recent report that TPA can down-regulate the signal transduction processes triggered by activation of the antigen receptor (1).

Our results indicate that differential phosphorylation is involved in the generation of the two *ets-2* components p56 and p54. This does not, however, lead to the conclusion that the diffuse p56 band consists solely of the phosphorylated form of p54. Quantification by densitometric analysis of the mitogen-induced conversion of p54 to p56 (data not shown) suggested that the lower part of the broad p56 band also shifts to a slightly higher position, thus giving p56 a sharper appearance upon stimulation. The result of acid phosphatase treatment showed that the bottom part of p56 may contain an unphosphorylated species (Fig. 2C). Previously, Boulukos and others (4) identified four species of chicken *ets-2* proteins, among which two are the phosphorylated forms of the other two species. They also detected two species of the human *ets-2* protein (4).

The multifunctional Ca^{2+}-calmodulin-dependent protein kinase is one of the major protein kinases activated by Ca^{2+} (19). Although direct evidence has yet to be obtained to support the involvement of this enzyme in *ets-2* protein phosphorylation, the deduced amino acid sequence of the human *ets-2* protein contains a putative consensus site (Arg-X-X-Ser [for a review, see reference 19]) for phosphorylation by this enzyme (Arg-308–Val–Pro–Ser-311). This sequence is exactly conserved in the *ets-1* protein, and recently we found that the purified human *ets-1* protein can be phosphorylated in vitro by the multifunctional Ca^{2+}-calmodulin-dependent protein kinase (R. J. Fisher, S. Koizumi, S. Fujiwara, N. K. Bhat, and T. S. Papas, manuscript in preparation). In vitro phosphorylation experiments with purified *ets-2* protein are in progress. Careful examination of the effect of selective inhibitors of calmodulin will be required to demonstrate the involvement of this enzyme.

Previously, Pognonec and others (14) reported that the chicken and mouse *ets-1* proteins in thymocytes are phosphorylated in response to a lectin mitogen and a Ca^{2+} ionophore. They also indicated that phosphorylation of the *ets-2* protein in a chicken macrophage line could be enhanced by a Ca^{2+} ionophore, although the biological significance of the Ca^{2+} signal in macrophages was not discussed

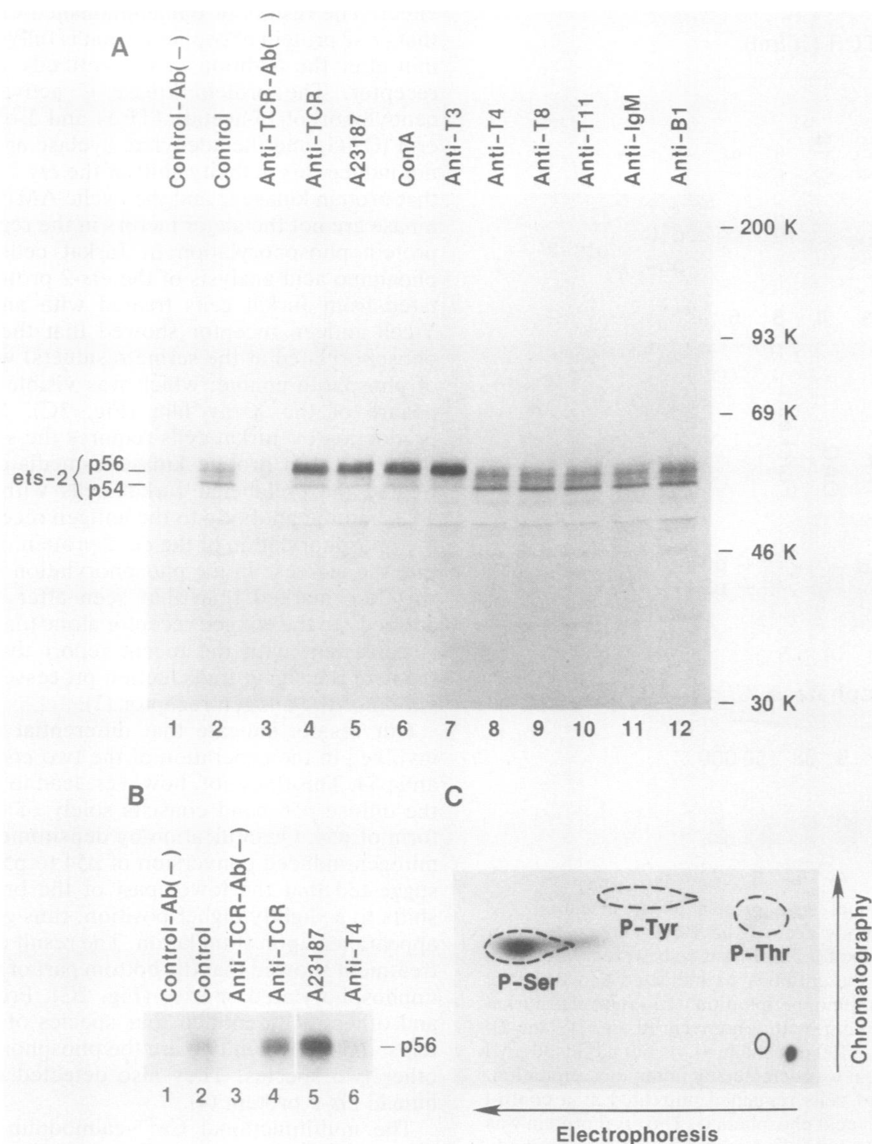

FIG. 3. Phosphorylation of *ets-2* protein in response to mitogenic signals. (A) Jurkat cells were prelabeled with [^{35}S]methionine (100 μCi/ml) for 3 h and treated briefly (10 min) with the following antibodies or mitogens before lysate preparation and immunoprecipitation. Lanes: 1 and 2, no treatment (control); 3 and 4, antibody to T-cell antigen receptor (1 μg/ml); 5, Ca^{2+} ionophore A23187 (1 μM); 6, concanavalin A (10 μg/ml); 7, antibody to OKT3 (1 μg/ml); 8, antibody to OKT4 (1 μg/ml); 9, antibody to OKT8 (1 μg/ml); 10, antibody to OKT11 (1 μg/ml); 11, antibody to immunoglobulin M (1 μg/ml); 12, antibody to B1 (1 μg/ml). Immunoprecipitation with the *ets-2* monoclonal antibody (lanes 2 and 4 through 12) or control antibody (lanes 1 and 3) and SDS-polyacrylamide gel electrophoresis were performed as described previously (7, 8). Mobilities of the [^{14}C]methylated protein size markers are shown on the right side of the panel. (K, $\times 10^3$). (B) Jurkat cells were prelabeled with $^{32}P_i$ for 4 h and treated with the following agents before lysate preparation and immunoprecipitation. Lanes: 1 and 2, no treatment (control); 3 and 4, antibody to T-cell antigen receptor (1 μg/ml); 5, A23187 (1 μM); 6, antibody to OKT4 (1 μg/ml). Immunoprecipitation was done with *ets-2* monoclonal antibody (lanes 2 and 4 through 6) or with control antibody (lanes 1 and 3). (C) Phosphoamino acid analysis of the *ets-2* protein. The origin of electrophoresis is indicated by the letter O, and the positions of phosphoamino acid standards are shown by circles. The directions of electrophoresis and chromatography are indicated.

(14). We have also found that the human *ets-1* protein, as well as *ets-2*, is phosphorylated by mitogenic stimuli such as antibodies against the T-cell antigen receptor-CD3 complex in a Ca^{2+}-dependent manner (S. Fujiwara, unpublished data).

Phosphorylation of the *ets-2* protein in response to activation of the antigen receptor suggests a possible role of the protein as a mediator of the signals at a certain step between the receptor activation and gene regulation. Its localization in the nucleus and properties similar to those of other proto-oncogene-encoded transcription factors (7) are consistent with the hypothesis that the *ets-2* protein is involved in the early phase of the gene regulation linked to Ca^{2+}-mediated signal transduction in T-lymphocyte activation. We have previously reported that activation of protein kinase C increases the half-life of the *ets-2* protein in human T-lymphocyte lines (7). Thus, it is now demonstrated that the *ets-2* protein responds posttranslationally to both Ca^{2+} and protein kinase C, the two interacting elements of signal transduction essential in T-cell activation.

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