collaboration with the intermediate and terminal glycosyltransferases. Since sLe^x determinant-bearing gp150 was not detected after TPA treatment for 5 days, the minimal detection of sLe^x antigen determinant might be due to greater sensitivity of flow cytometry analyses than immunoblot.

Following transfection and permanent expression of the C2GnT gene, we could remodel the sLe^x structure synthetic machinery from phorbolester-responsive to phorbolester-resistant. In the case of CD43, leukosialin, however, C2GnT gene transfection could only result in remodeling CD43 *O*-glycans from tetrasaccharides to hexasaccharides, from the 110- to 135-kDa glyco-form (23). In addition, only minor portions of *O*-linked oligosaccharides have been demonstrated to have poly-*N*-acetyllactosaminyl extensions and sLe^x structures in CD43 glycoprotein (37). Moreover, molecular size of our gp150 was distinct from that of CD43, and expression of sLe^x antigen determinants was significantly influenced by C2GnT gene transfection in KM3 cells during TPA treatment. Despite the overexpression of C2GnT transcript and enzyme activities, however, further augmentation of cell surface sLe^x expression could not be detected in nontreated KM3c1e2 cells. This may be because C2GnT level is saturated for influencing the sLe^x synthetic machinery in nontreated KM3c1e2 cells or may be due to some other inhibiting mechanisms on the machinery.

Together with determinative action of FucT-VII, C2GnT has been reported to play an important role for presenting sLe^x on P-selectin glycoprotein ligand-1 (PSGL-1) (38). However, we could not detect PSGL-1 transcript in pre-B KM3 cells (data not shown). The size of PSGL-1 is 250 kDa in nonreduced form and 120 kDa in its reduced condition (39), and it is different from that of our gp150 in KM3 cells. In addition, the recent report said that PSGL-1 is essential for adhesion to P-selectin but not E-selectin (33). Therefore, E-selectin-mediated cell adhesion of pre-B KM3 cells presented in this report may be distinct from the one through PSGL-1. Moreover, a direct and dynamic involvement of a single glycosyltransferase, C2GnT, in the control of CD15s expression during differentiation with constitutive and static FucT-VII expression has not been elucidated in any cell systems so far.

The molecular size of gp150 was also different from those of L-selectin (~76 kDa) and lysosomal membrane glycoproteins (lamp-1 and lamp-2) (~115 kDa) (40, 41). Lymphocyte L-selectin was not suggested to react with E-selectin-transfected cells (42), and lamp-1 and lamp-2 were reported to carry sLe^x structures on *N*-glycans but not on *O*-glycans (43). Therefore, it is less possible that our gp150 is identical to one of those previously known core proteins. On the other hand, gp150 could be a human counterpart of murine E-selectin ligand-1, judging from the molecular size (44). However, E-selectin ligand-1 is suggested to be *N*-glycosylated and at most only weakly *O*-glycosylated (44). By contrast, our gp150 is suggested to be essentially *O*-glycosylated. Therefore, further identification and characterization of gp150 including direct E-selectin accessibility to the glycoprotein is highly necessary.

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Identification of Alternatively Spliced Transcripts Encoding Murine Macrophage Colony-Stimulating Factor

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We have isolated a novel cDNA encoding macrophage colony-stimulating factor (M-CSF) from a murine stromal cell line, ST2. The cDNA included an entire coding sequence of the M-CSF gene but contained an additional sequence of 140 base pairs (bp). Northern blot analysis demonstrated that other murine cell lines such as a fibroblastic cell line (L) and a stromal cell line (PA6) also expressed the transcripts corresponding to the clone. The nucleotide sequence analyses of the cDNA and the cloned M-CSF genome revealed that the 140-bp insertion sequence was part of intron 1 which separated exon 1 and exon 2: the former contained part of the amino acid residues of the signal sequence and the latter the rest of the signal sequence and the first 22 amino acid residues of the mature protein. The insertion of the 140-bp intron sequence not only changed the amino acid sequence of the signal peptide but also generated an in-frame termination codon. However, instead of the dysfunction of the original initiation codon, the 140-bp insertion sequence contained a putative ATG initiation codon that preserved the original open reading frame. Finally, we found that the cDNA directed the expression of a secreted and biologically active M-CSF protein when it was introduced into COS7 cells and M-CSF activity in the culture supernatants was measured using an M-CSF-dependent cell line. These results indicate the presence of an alternatively spliced M-CSF transcript which utilizes an alternate initiation codon in order to specify active M-CSF protein.

Macrophage colony-stimulating factor (M-CSF) is a regulatory cytokine that stimulates the proliferation, differentiation, and survival of monocytes/macrophages and their committed progenitor cells (1). The expression of M-CSF receptor and the action of M-CSF have been thought to be restricted to monocyte/macrophage lineage in hematopoietic systems (2, 3). However, we and other investigators recently reported that M-CSF had an ability to develop bone marrow fibroblastic stromal cells (4-6). Although it remains to be elucidated whether or not M-CSF acts directly on the progenitor of the stromal cells, this is a novel function of M-CSF (5, 6).

The human M-CSF gene is comprised of 10 exons and 9 introns spanning 20 kilo bases (kb) of the genome (7), which is presumably conserved in murine systems (8). Although M-CSF is transcribed from a single gene (7, 9), it is well known that multiple splice variants of the transcript are produced from the gene (7, 9-11). For example, in the original isolation of human M-CSF cDNA from a pancreatic carcinoma cell line, phorbol myristate acetate was shown to induce at least seven M-CSF transcripts ranging from 1.5 to 4.5 kb (10). At least four different forms of M-CSF cDNA have been isolated and sequenced (7, 9-13). In addition to the complexity in the transcriptional regulation of the M-CSF gene, the translational and posttranslational modification of the primary products derived from the multiple species lead to the production of several different protein forms, e. g., a secreted and a membrane-associated form.

The secreted form is encoded by two transcripts of 2.5 and 4.0 kb, which differ only in their 3′ noncoding region: the 2.5 kb- or 4.0 kb transcript uses exon 9 or 10, respectively, of the M-CSF gene (7, 9, 11-15). They contain the complete exon 6 encoding an amino acid sequence which should be cleaved by proteases, leading to the rapid secretion of the protein from the cell (14, 15). In spite of the presence of a single open reading frame, these transcripts specify at least two protein species, i. e., an M-CSF protein with the Mr of 85,000 and that with the Mr greater than 200,000 (11, 12, 14-16). The latter was uniquely modified by a chondroitin sulfate glycosaminoglycan chain (16-18). Due to this modification, this type of M-CSF can bind to type V collagen, and this might allow the species to associate with extracellular matrix (19, 20). The other two transcripts of 1.6 and 3.1 kb encoded the M-CSF protein

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associated with the cell membrane (7, 10, 12, 21). These transcripts again differ only in the alternate use of the 3′ nonconding region, i. e., exon 9 or 10 (7, 10, 12, 21). In these transcripts, the splicing out of the large segments of exon 6 deletes a region of 298 amino acid residues containing the site at which proteolytic cleavage occurs before the secretion of the active M-CSF described above. Therefore, the product from the transcripts is stably expressed at the cell surface and is also biologically active (14, 22).

Although the 4.0 kb form is predominant in many cells (7, 9-12), all the available evidence suggests to us that there are splice variants which are yet to be fully characterized. The cloning of minor splice variants is therefore necessary to clarify the significance of such species, if any. In this paper, we report the identification and characterization of a novel alternatively spliced M-CSF transcript.

EXPERIMENTAL PROCEDURES

Cell culture. Murine bone marrow stromal cell lines, ST2 (23) and MC3T3-G2/PA6 (PA6; 24), and the murine fibroblastic cell line L were obtained from the Riken Cell Bank (Tsukuba, Japan). They were routinely maintained in DMEM medium (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). Monkey kidney COS7 cells (Riken Cell Bank) were also routinely cultured with DMEM medium containing 10% FCS. Murine M-NFS-60 cells (25), which are dependent on the presence of M-CSF for their proliferation and were used for the determination of M-CSF activity in this study, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were routinely maintained in RPMI1640 medium (Life Technologies) containing 10% FCS and recombinant human M-CSF at a concentration of 100 ng/ml (26).

Polymerase chain reaction (PCR). Total RNA was isolated from a subconfluent culture of ST2, PA6, or L cells by using RNA zol B reagent (TEL-TEST, Friendswood, TX) (27). One microgram of total RNA was reverse transcribed by using 2.5 mM random 9 mers, 2.5 U Avian Myeloblastosis Virus reverse transcriptase, and reagents from a commercial RNA PCR kit (RNA LA PCR Kit; TaKaRa Shuzo, Kyoto, Japan) (28). The PCR amplification was performed by using LA Taq polymerase (TaKaRa Shuzo) and according to the recommendations of the manufacturer (28). For the amplification of the entire coding region of M-CSF, two primers (P1 and P2) were generated, which were based on the previously described murine cDNA sequences (12). P1 hybridized to nucleotide sequences 139-163 in exon 1, and its sequence was 5′-TGCCGGGACCCAGCTGCCCGTATGA-3′. P2 was anti-sense to nucleotide sequences 1801-1825 in exon 8, and its sequence was 5′-TCCTTTCTATACTGGCAGTTCCACC-3′. Thus, the primer pair P1/P2 would generate at least two fragments which were derived from the transcript containing the complete exon 6 and that containing the truncated exon 6 (12). The PCR conditions were as follows: 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C and 2 min of extension at 72°C (28). Genomic DNA was also isolated from ST2 cells by using a reagent containing SDS and protease (GENOME DNA Isolation Kit; BIO 101, Vista, CA) (29). The PCR amplification of the region including intron 1 of M-CSF genome was performed by using the DNA as the template, LA Taq polymerase (TaKaRa Shuzo), and two oligonucleotide primers (P1 and P3). The primer P1 hybridized to nucleotides in exon 1 as described above. P3 was anti-sense to nucleotide sequences 226-250 in exon 2; its sequence was 5′-TACTCCTGCTCATGAGGAGACAGAC-3′ (12). The PCR conditions were as follows: 20 sec of denaturation at 98°C, and 3 min of annealing and extension at 68°C (28). The analysis of PCR products was performed by comparing the predicted PCR fragment length with the actual PCR products after the ethidium bromide staining of products separated by electrophoresis on a 1 or 2% Seakem GTG agarose gel (FMC BioProducts, Rockland, ME).

Cloning and sequencing of PCR products. The PCR products were ligated directly into a pCR 2.1 vector (Invitrogen, Carlsbad, CA) (30) and sequenced by the dye terminator cycle sequencing method (Perkin Elmer, Foster City, CA). Sequencing was carried out in both orientations of the cDNAs, using an M13 reverse primer, M13 forward primer (29), and appropriately synthesized primers which hybridized to the sequences of the templates.

Northern blot analysis. Total RNA was prepared as described above. The RNA (10 μg/lane) was fractionated by electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde (29). The size-fractionated RNA was transferred from the gel to a nylon membrane (Hybond N+; Amersham, Buckinghamshire, England) and fixed by baking. The resulting filter was probed with cDNAs labeled with [^{32}P]dCTP (ICN Biomedicals, Irvine, CA), using the random oligonucleotide priming reaction (MegaPrime DNA Labelling Systems; Amersham). The hybridization was performed at 65°Cusing rate-enhanced hybridization solution (Rapid-hyb buffer; Amersham). The filters were washed with 2x SSC (1x SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at room temperature, and the final washings were done with 0.1× SSC containing 0.1% SDS at 65°C (29). The relative mobilities of different species of M-CSF mRNA were visualized by exposure of the filters to Hyperfilm MP (Amersham).

Transfection and analysis of M-CSF. The cDNA fragments containing a putative open reading frame of M-CSF were subcloned into a mammalian expression vector, pCEF. The vector is a derivative of pRc/CMV (Invitrogen, San Diego, CA), which we made by the inserting HindIII/XbaI fragment of a vector pEF-BOS containing the promotor region of polypeptide chain elongation factor-1 (31) into the pRc/CMV vector. The resulting plasmid DNAs were transfected into COS7 cells by using LipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions (32). The media were removed 72 hrs later and assayed for the concentration of M-CSF by measuring the proliferation of M-CSF-dependent M-NFS-60 cells (25, 26). In brief, cells were seeded into 96-well culture plates at a concentration of 1×10^4 cells per well and incubated for 44 hrs in the presence or absence of experimental samples. The recombinant murine M-CSF (R&D Products, Minneapolis, MN) was also included in the assay. Following the addition of [^{3}H]thymidine (ICN Biomedicals), its incorporation into DNA was measured after a 6-h period (26).

RESULTS

Identification and cloning of alternatively spliced M-CSF transcript. We performed a reverse transcription PCR (RT-PCR) analysis using total RNA obtained from murine bone marrow stromal cells (ST2), which were shown to constitutively produce M-CSF (6). As shown in Fig. 1, the RT-PCR reaction using primers P1 and P2 resulted in the amplification of four different products, whose sizes were approximately 1850, 1700, 950, and 800 base pairs (bp). The primers were chosen so as to amplify the entire coding region of M-CSF, and so that the primer pair would generate fragments with sizes of 1686 or 800 bp. The former would be derived from the transcript containing the complete exon 6, and the latter from the transcripts containing the truncated exon 6 (12). Thus, the fragments of approximately 1850

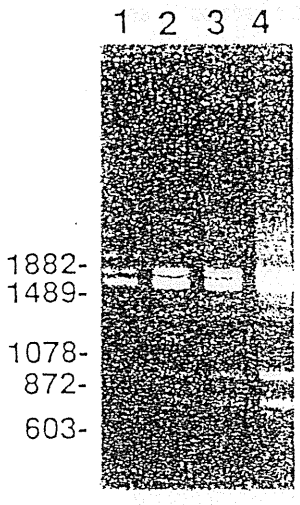


FIG. 1. RT-PCR amplification of fragments derived from M-CSF transcripts. An RT-PCR was performed by using total RNA from ST2 cells and the primer pair P1/P2. An aliquot was removed from the reaction mixture at the ends of 28 cycles (lane 1), 30 cycles (lane 2), 32 cycles (lane 3) and 34 cycles (lane 4) of the PCR amplification. The amplified products were then analyzed by electrophoresis on 2% agarose gel and visualized after ethidium bromide staining of the gel. The molecular mass standards are shown at the *left* in bp.

and 950 bp were unexpected amplification products in the RT-PCR reaction. The Southern blot analysis with M-CSF cDNA as a probe revealed that these additional fragments actually contained an M-CSF-related sequence (data not shown). We therefore cloned these two fragments together with the expected amplification products and analyzed their nucleotide sequences.

The DNA sequence analysis of the cDNAs derived from the two additional fragments revealed that both possessed an insertion of a 140-bp sequence in the coding region (indicated by the underline in Fig. 2A). In addition, the difference in length between the additional two fragments was demonstrated to be due to the result of an alternate use of exon 6 that had been reported (12): the longer fragment contained the complete exon 6, whereas the shorter contained the truncated form of exon 6. We also found that the 140-bp sequence was inserted at the junction of the exon 1-derived sequence and the exon 2-derived sequence (7, 12, 33). The published sequence of murine M-CSF demonstrated that exon 1 encoded the 5′ untranslated region and the first 13 amino acid residues of the signal sequence, and exon 2 encoded the remaining 19 amino acid residues of the signal sequence and the first 22 amino acid residues of the mature protein (7, 12, 13, 21, 33). The insertion of the 140-bp sequence resulted in the generation of TGA stop codon 31 bp downstream from the beginning of the inserted sequence (Fig. 2A). However, there were repetitive ATG codons in the inserted sequence, which overlapped the TGA stop codon (indicated by the boldface in Fig. 2A). If the ATG codon is used as a translational initiation codon, the reading frame of M-CSF is preserved in these cDNAs, though the first 13 amino acid residues of a 32-amino acid signal peptide will be replaced with an unrelated sequence of 37 amino acid residues in the cDNAs (indicated by the underline in Fig. 2B).

We next examined the origin of 140-bp insertion sequence. For this purpose, we performed a PCR amplification of the M-CSF genome containing intron 1 that separated two exons, i.e., exons 1 and 2. An approximately 3.1 kb fragment was detected (Fig. 3A), which would be expected based on the reported intron-exon structure of the human M-CSF gene (7, 33). The fragment was therefore cloned and the genomic DNA was analyzed by sequencing. As a result, we found that the 140-bp insertion sequence was part of intron 1. It was located near the 3′ end of the intron (indicated by the parentheses in Fig. 3B). We therefore tentatively designated the 140-bp sequence exon 1.1. The 5′ end of exon 1.1 was preceded by a possible acceptor sequence (indicated by the boldface in Fig. 3B), and the 3′ end was followed by a possible donor sequence (indicated by the italics in Fig. 3B) as discussed below.

The cDNA containing the entire coding region of M-CSF (12) and the cDNA fragment isolated from the region within exon 1.1 were each used as a probe to examine the expression of M-CSF transcript. The Northern blot analysis of ST2 cells and two additional two cell lines (i. e., the murine bone marrow stromal cell line PA6 and the murine fibroblastic cell line L) with the wild-type cDNA revealed a major hybridizing species of approximately 4.0 kb as well as a minor species of approximately 2.5 kb (Fig. 4, lanes 1-3). The longer exposure of the blot revealed the presence of at least four less- abundant species ranging from 1.5 to 4.5 kb (data not shown). Hybridization of another blot with the cDNA fragment specific to the sequence of exon 1.1 established that all three cell lines expressed the M-CSF transcript possessing exon 1.1 (Fig. 4, lanes 4-6). The size of the transcript was indistinguishable from that of a major species detected on the blot probed with the wild-type cDNA. The Northern blot analysis was performed under conditions of high stringency, as described in Experimental Procedures (29). Taken together with the finding that exon 1.1 was only part of intron 1 (Fig. 3B), the M-CSF transcript containing the exon 1.1 sequence was demonstrated to be due to an alternative splicing event.

Expression of M-CSF cDNA in COS7 cells. Finally, we investigated whether the M-CSF transcript containing exon 1.1 could direct the production of biologically active M-CSF protein. Initially, we made four plasmids which carried different constructions of the cDNA insert, i. e., the cDNA containing exon 1.1 and the complete exon 6 ("CSF-A" in Fig. 5), that containing the complete exon 6 but not exon 1.1 ("CSF-B"), that

A TGCCGGGACCCAGCTGCCCGT*ATG*ACCGCGCGGGGCGCCGCGGGGCGCTGCCCTTCTTCG 60

TGGATCCAGGTTCAAATCCCAGAATCTAGATGATGGTTCACAACCATTCATACCTCCAGG 120

TCCAGGAGATTCCAAGAAGGAAGCACATAGAGTGTGTGCATGCAAGCAGGCAAAATATTC 180

ATAAACACAAAACAAAAACAACATGGCTGGGCTCCCGGCTGCTGCTGGTCTGTCTCCTCA 240

TGAGCAGGAGTATTGCCAAGGAGGTGTCAGAACACTGTAGCCACATGATTGGGAATGGAC 300
T
ACCTGAAGGCCCTGCAGCAGTTGATCGACAGTCAAATGGAGACTTCATGCCAGATTGCCT 360

TTGAATTTGTAGACCAGGAACAGCTGGATGATCCTGTTTGCTACCTAAAGAAGGCCTTTT 420

TTCTGGTACAAGACATAATAGATGAGACCATGCGCTTTAAAGACAACACCCCCAATGCTA 480

ACGCCACCGAGAGGCTCCAGGAACTCTCCAATAACCTGAACAGCTGCTTCACCAAGGACT 540

ATGAGGAGCAGAACAAGGCCTGTGTCCGAACTTTCCATGAGACTCCTCTCCAGCTGCTGG 600

AGAAGATCAAGAACTTCTTTAATGAAACAAAGAATCTCCTTGAAAAGGACTGGAACATTT 660

TTACCAAGAACTGCAACAACAGCTTTGCTAAGTGCTCTAGCCGAG ---------

B MetMetValHisAsnHisSerTyrLeuGlnValGlnGluIleProArgArgLysHisIle 20

MetThrAlaArgGlyAlaAlaGlyArgCysProSerSer
GluCysValHisAlaSerArgGlnAsnIleHisLysHisLysThrLysThrThrTrpLeu 40

GlySerArgLeuLeuLeuValCysLeuLeuMetSerArgSerIleAlaLysGluValSer 60
Val
GluHisCysSerHisMetIleGlyAsnGlyHisLeuLysAlaLeuGlnGlnLeuIleAsp 80

SerGlnMetGluThrSerCysGlnIleAlaPheGluPheValAspGlnGluGlnLeuAsp 100

AspProValCysTyrLeuLysLysAlaPhePheLeuValGlnAspIleIleAspGluThr 120

MetArgPheLysAspAsnThrProAsnAlaAsnAlaThrGluArgLeuGlnGluLeuSer 140

AsnAsnLeuAsnSerCysPheThrLysAspTyrGluGluGlnAsnLysAlaCysValArg 160

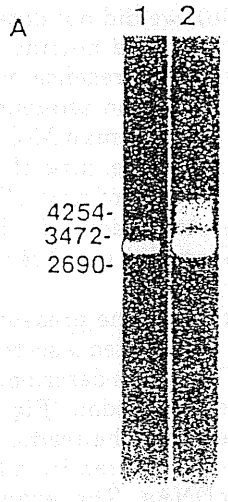
ThrPheHisGluThrProLeuGlnLeuLeuGluLysIleLysAsnPhePheAsnGluThr 180

LysAsnLeuLeuGluLysAspTrpAsnIlePheThrLysAsnCysAsnAsnSerPheAla 200

LysCysSerSerArg ---------

FIG. 2. DNA sequence and deduced amino acid sequence of PCR products. The amplified fragments of approximately 1850 and 950 bp shown in Fig. 1 were cloned and the cDNA inserts were analyzed by sequencing. (A) The 140-bp insertion sequence not found in the reported cDNAs is underlined. The initiation codon which is thought to be utilized in the reported cDNAs is indicated by italics. The in-frame ATG codons within the 140-bp insertion sequence are indicated by boldface. The nucleotide difference between this sequence and the sequence reported by Ladner et al. (12) is shown above the nucleotide sequence (T → C at nucleotide position 310). The change was also observed in the cDNA clones derived from the amplified fragments of 1700 and 800 bp shown in Fig. 1, which represented the reported structure of M-CSF transcript. This change alters the amino acid (Val → Ala) as shown in Fig. 2B. In any case, the single amino acid change was not critical to the function of the protein, as shown in Fig. 5. The cDNAs derived from the fragments of approximately 1850 and 950 bp differed in the structure of exon 6, as reported with the previously isolated cDNAs (12). Since the sequence downstream exon 6 of the isolated cDNAs agrees with that reported by Ladner et al. (12), we omitted the nucleotide sequence as well as the deduced amino acid sequence for that portion. (B) The presumed amino acid sequence by the isolated cDNA is depicted. The amino acid sequence not found in the reported cDNAs is underlined. The sequence deduced by the reported cDNAs is shown above this sequence (12). The first amino acid in the mature M-CSF protein (Lys) in indicated by the boldface.

containing exon 1.1 and the truncated exon 6 ("CSF-C"), and that containing the truncated exon 6 but not exon 1.1 ("CSF-D"). These plasmids were introduced into COS7 cells, and the media conditioned by the transfected cells were assayed for the presence of biologically active M-CSF using an M-CSF-dependent M-NFS-60 cell line. As shown in Fig. 5, the plasmids containing exon 1.1 ("CSF-A" and "CSF-C") directed the



B

exon 1

TGCCGGGACCCACTGCTGCCCGTATGACCGCGCGGGGGCGCCGCGGGGCGCTGCCCTTCTTCG]
GTAAGCTGCAACCGTGGCGCGCGGGGCCCGGGCCGGGCTGGGGCAGGAGCTCTGCAGCAA
GCAGCAGGCGGCTCTGCGGGGCCACTTGGAGCGGACAGCCCCTTCTCGCCAGCTGCCCAG
GCTTCTCGGCCCTGGGTTCTGGCTTCCTTACTGCTTCTAAGCTACGGCTGAGGCCCGCGG
TATTTCAATAGCTGCTCCTGGGGCTGCAGCGCTTTGCGAGGTAAAGAGAAGGCTGCTCAT
CCCATTGCACGGAGGAGAACACTGAGCTACCCACAGGCTGGA----------TGTAA

exon 1.1

GATGGAAAGGTGGCTCATCATTTAAAAACACTTGTTGCTCTTGCAG [TGGATCCAGGTT
CAAATCCCAGAATCTAGATGATGGTTCACAACCATTCATACCTCCAGGTCCAGGAGATTC
CAAGAAGGAAGCACATAGAGTGTGTGCATGCAAGCAGGCAAAATATTCATAAACACAAAA
CAAAAACA]GTAAGCCCTTTTAAAATTGTCTTTTAAAGTACTCTGTATTGTTGAGCTGCT
CTGGGTTAACCTCAGAAGAGTTCTTGGAGAAGATGGCATTTGGGCTATTACCAAGGGAAG
AGTGAGGTGGTGCCCTGGCTGATAAGAATCTAATGGTGCTGCAGCATGGGAATGGGGCGT
TGTTTCTTTGCCCTTAGTGGGACATGGGGGTAGCTAGGGAGAGGAAGTGCTGGAAAGACA

exon 2

TGACTCCCTTCTGTTATAG [ACATGGCTGGGCTCCCGGCTGCTGCTGGTCTGTCTCCT
CATGAGCAGGAGTA

FIG. 3. PCR amplification of the genomic region containing exon 1 of M-CSF gene and partial characterization of the genomic clone. (A) The PCR amplification was performed by using genomic DNA from ST2 cells and the primer pair P1/P3. An aliquot was removed from the reaction mixture at the ends of 25 cycles (lane 1) and 30 cycles (lane 2) of the PCR amplification. The amplified products were then analyzed by electrophoresis on 1% agarose gel and visualized after ethidium bromide staining of the gel. The molecular mass standards are shown at the *left* in bp. (B) The amplified fragment shown in Fig. 3A was cloned and the cDNA inserts were analyzed by sequencing. The 140-bp insertion sequence, which we designated exon 1.1, is indicated by parentheses. The possible acceptor sequence and donor sequence for exon 1.1 are indicated by the boldface.

production of biologically active M-CSF into the media, as did those without exon 1.1 ("CSF-B" and "CSF-D"), although the activities in the media conditioned by COS7 cells transfected with the plasmids containing exon 1.1 were somewhat lower than those obtained by using the plasmids without the exon. As mentioned above (Fig. 2A), the original ATG codon did not specify the M-CSF protein in the cDNAs containing exon 1.1. Therefore, the result shown in Fig. 5 indicated that an alternate initiation codon was functionally utilized in the cDNAs. When we removed the complete sequence of exon 1 which included the original ATG codon from the cDNAs with exon 1.1 and introduced the resulting plasmids ("dCSF-A" and "dCSF-C" in Fig. 5) into COS7 cells, the result of the bioassay showed that these truncated cDNAs also directed the expression of active M-CSF, supporting our conclusion described above.

DISCUSSION

We have shown that murine ST2 cells express an M-CSF transcript that has not been previously described. The transcript contained the sequence within intron 1 (Figs. 2 and 3). The presence of this transcript was confirmed by Northern blot analysis (Fig. 4B). Since only part of the intron sequence was found, the transcript seemed to be due to an alternative splicing event and not simply to incomplete splicing. An examination of the sequences surrounding the inserted sequence, which we designated exon 1.1, might support this hypothesis. The 3′ end of exon 1.1 was followed by a possible donor sequence (GTAAGC, Fig. 3B) (34), which was similar to the sequence at the intron/exon boundaries of the human M-CSF gene (7). The 5′ end of exon 1.1 was preceded by a possible acceptor sequence (CTCTTGCAG, Fig. 3) (34). However, the site had a short pyrimidine tract, a critical determinant of the strength of splice acceptors (35), and

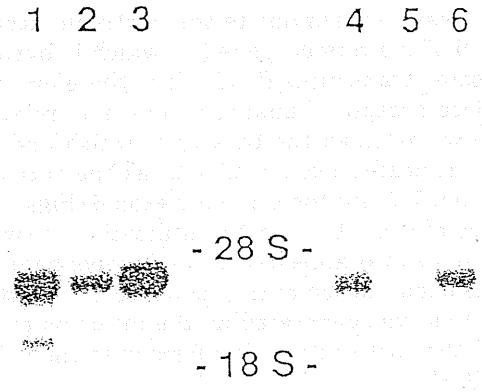


FIG. 4. Northern blot analysis of M-CSF transcripts. Total RNA from ST2 cells (lanes 1 and 4), PA6 cells (lanes 2 and 5), and L cells (lanes 3 and 6) was analyzed for the presence of M-CSF transcript by Northern blot analysis. The hybridization was performed with the cDNA insert obtained by cloning the fragment of approximately 1700 bp shown in Fig. 1, which represented the reported structure of M-CSF transcript (lanes 1-3), or the cDNA fragment whose sequence was specific to exon 1.1 (lanes 4-6), as a probe. The latter was prepared from the cDNA with exon 1.1 by using the restriction enzymes BamHI and SspI. Markers are mammalian ribosomal RNAs.

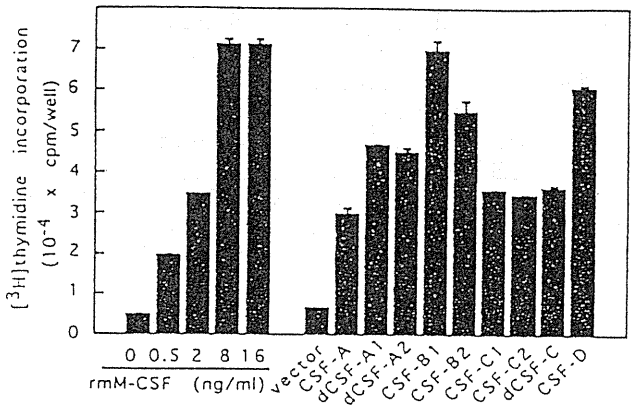


FIG. 5. M-CSF activities in the media conditioned by COS7 cells transfected with M-CSF cDNAs. The cDNA inserts were constructed as follows. The four different fragments amplified by RT-PCR shown in Fig. 1 were initially cloned into pCR2.1 vector. The cDNA inserts were then excised from the vector and recloned into the vector pCEF. Therefore, there were four types of the cDNA insert, i.e., the cDNA containing exon 1.1 and the complete exon 6 (CSF-A), that containing the complete exon 6 but not exon 1.1 (CSF-B), that containing exon 1.1 and the truncated exon 6 (CSF-C), and that containing the truncated exon 6 but not exon 1.1 (CSF-D). We also made two types of deletion mutants (dCSF-A and dCSF-C). This was accomplished by the removal of the 5′ portion of CSF-A or CSF-C cDNA using a restriction enzyme XbaI, which deleted the first 89 nucleotides shown in Fig. 2A. The pCEF vector and the resulting plasmids were introduced into CoS7 cells. The number of the cDNA represents the independent isolation of the plasmids. The media conditioned by COS7 cells were assayed for M-CSF activity after a 500-fold dilution using M-NFS-60 cells. The murine recombinant M-CSF was also included in the assay at the indicated concentration.

its sequence was very similar to the upstream acceptor site of exon 6 of the human gene (7), which is bypassed in at least some transcripts (9-13). It is therefore likely that the splice acceptor flanking exon 1.1 is relatively weak. Since we detected the transcript which had exon 1.1 and the truncated exon 6 as well as the transcript which had exon 1.1 and the complete exon 6 (Figs. 1 and 2), the choice of exon 1.1 and the utilization of two acceptor sites of exon 6 appeared to be independent phenomena. The examination of the sequence of the ends of two introns that was generated by the insertion of exon 1.1 revealed that both introns conformed to the GT-AG rule (Fig. 3B) (35).

The expression of the transcript with exon 1.1 was not specific to ST2 cells. As shown in Fig. 4B, other murine cell lines including PA6 and L also expressed the M-CSF transcript possessing the exon. We confirmed this result by performing an RT-PCR with total RNA obtained from these cells using the primer pair P1/P2 (data not shown). We did not obtain an evidence for the presence of human equivalents for the murine transcript. In an experiment I which we performed an RT-PCR with RNA obtained from a human stromal cell line, KM102, human umbilical vein endothelial cells, human peripheral monocytes or a human osteoblastic cell line, MG63 (26,36). we did not detect the amplified product corresponding to the murine transcript (data not shown). However, the presence of a human transcript which carried an intron sequence was reported. In the original isolation of human M-CSF cDNA from a pancreatic carcinoma cell line, nine of ten cDNA clones isolated contained a 115 bp of part of intron 2, which includes an in-frame stop codon (10). It was also demonstrated that this form did not direct the production of an active protein (10).

The intriguing finding in the present study was that the cDNA with exon 1.1 encoded a secreted- and biologically active M-CSF (Fig. 5). As described above, exon 1.1 included an in-frame stop codon (Fig. 2A). Therefore, these results indicated that the translational initiation codon used by the cDNA differed from that used by the previously isolated cDNAs. The examination of previously reported sequences revealed that there were two in-frame ATG codons between the transcriptional starting point (33) and the codon corresponding to the amino terminus of mature M-CSF protein (37, 38). The upstream ATG is an initiation codon, because the downstream ATG was not found in the human gene (7, 10, 11). Furthermore, the utilization of the downstream ATG caused a significant loss of the signal peptide, especially a complete loss of the region being rich in hydrophobic amino acids (ThrTrpLeuGlySerArgLeuLeuLeuValCysLeuLeu, in Fig. 2B), which is a characteristic of known signal peptides (39). If the ATG codons within exon 1.1, which preserves the reading frame of M-CSF as described above (Fig. 2B) is utilized as an initiation codon, the first 13 amino acid residues of a 32-amino acid signal sequence are replaced with an unrelated sequence comprised of 37 amino acid residues (Fig. 2B). However, this change still preserves the hydrophobic core sequence mentioned above, which might allow the translational product by the cDNA with exon 1.1 to be secreted (Fig. 5). The M-CSF activities in the media conditioned by COS7 cells transfected with the plasmids containing exon 1.1 were lower than those obtained by using the plasmids containing the previously defined structure of M-CSF cDNA. This might be due to the difference in the sequence flanking the ATG initiation codon among the two types of cDNA, which could affect the translational efficiency (40). In any case, this is the first report demonstrating the functional utilization of the alternate initiation codon in M-CSF gene.

At least five groups of investigators have reported the sequences for a portion or a full coding region of murine M-CSF (9, 12, 13, 21, 33). However, the reported sequences differ slightly in exon 1. The sequences of the coding region within exon 1 are

ATGACCGCGCGGGGCCGCGCGGGGCCGTGCCCTTCTTCG (21)
** **
ATGACCGCGCGGGGCGCCCGCGGGGCGCTGCCCTTCTTCG (12, 13, 33)
**
ATGACC - CGCGGGCGGCCGC - - GGCGCTGCCCTTCTTCG (9)

The diversity of the sequences within this region might be due to an artifact of the sequencing analysis, because the region contains a stretch of GC residues which might be difficult to analyze. However, it is also possible that the region is the site at which mutations frequently occur. The presence of the M-CSF transcript which utilizes the alternate ATG codon might be a mechanism of escape from a possible inactivation of the M-CSF gene by such mutations.

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RAPID COMMUNICATION

Tec and Jak2 Kinases Cooperate to Mediate Cytokine-Driven Activation of c-*fos* Transcription

By Yoshihiro Yamashita, Sumiko Watanabe, Akira Miyazato, Ken-ichi Ohya, Uichi Ikeda, Kazuyuki Shimada, Norio Komatsu, Kiyohiko Hatake, Yasusada Miura, Keiya Ozawa, and Hiroyuki Mano

Although transcriptional activation of the c-*fos* proto-oncogene plays an intrinsic role in the mechanism of blood cell growth, it is still obscure how protein-tyrosine kinases (PTKs) regulate the cytokine-driven c-*fos* activation pathway. We present here that Tec PTK is tyrosine-phosphorylated and activated by granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in a human GM-CSF–dependent cell line. Moreover, we could show that introduction of Tec into mouse BA/F3-hGMRαβ cells can profoundly activate the c-*fos* promoter in response to GM-CSF or to interleukin-3 (IL-3). In contrast, introduction of a kinase-deleted Tec could suppress cytokine-driven c-*fos* activation, indicating that Tec is directly involved in the regulation of c-*fos* transcription. Interestingly, strong activation by Tec of the c-*fos* promoter was blocked by the co-expression of dominant negative Jak2. The molecular interaction between Tec and Jak2 was then investigated both in mammalian and insect cell systems, revealing that they can not only bind to each other, but either of the two can phosphorylate the other. Thus, Tec and Jak2 can "cross-talk" in a complexed way to mediate cytokine-driven c-*fos* activation.

TEC PROTEIN-TYROSINE kinase (PTK) is the prototype of a recently emerging subfamily among nonreceptor PTKs including Tec, Btk, Emt/Itk/Tsk, Txk, and Bmx.[1,2] In contrast to the Src-family kinases, none of the Tec-family members carry the myristylation signals or the C-terminal tyrosine residues corresponding to Tyr-527 in c-Src. One of the characteristic feature of the Tec-family members is that they (with the exception of Txk) contain a relatively long N-terminal unique region comprising a pleckstrin homology (PH) domain[3] and a Tec homology (TH) domain.[4] Tec-family members are, to date, the only PTKs containing the PH domain in their structures. Subsets of phospholipids have been shown to bind to the PH domain of Btk[5,6] and Tec (T. Shirai and Y. Fukui, personal communication), and this PH-phospholipid interaction is supposed to play an important role in the recruitment of the PTKs to cell membrane and/or in the regulation of the kinase activities, as already proven in the case of a serine/threonine kinase, c-Akt/PKB/Racα.[7]

Although physiological roles of the Tec-family members are still to be revealed, accumulating evidence has suggested that Tec-family PTKs may be involved in the growth and/or differentiation mechanism of hematopoietic cells. First, many Tec-family members are abundantly expressed in blood cells; for instance, Btk in myeloid cells and B lymphocytes,[8] Emt/Itk/Tsk in T lymphocytes,[9] and Tec in all the lineages.[2] Second, mutations in Btk cause agammaglobulinemia in humans.[10,11] Third, many Tec-family kinases have been shown to be implicated in the intracellular signaling pathways of cytokines. We and our colleagues have shown that Tec can be tyrosine-phosphorylated and activated in response to interleukin-3 (IL-3), IL-6, stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), or thrombopoietin (TPO).[12-17] Tec was shown to be physically associated (either directly or indirectly) with the receptors for SCF and IL-6. In addition to Tec, Btk and Emt/Itk/Tsk have also been shown to be activated by growth/differentiation signals of lymphocytes.[18-20] Thus, currently one of the major concerns in the research of Tec-family kinases is in which part of intracellular machinery of cytokines they directly participate.

The c-*fos* proto-oncogene is one of the immediate early genes induced by a wide range of cytokine stimulations, and can encode a transcription factor containing a "leucine zipper" structure.[21] Although transcriptional activation of c-*fos* is not directly involved in DNA synthesis, it is believed to be one of the important mechanisms to maintain the growth of blood cells.[22] So far, two PTK-subfamilies have been suggested to play a regulatory role in the c-*fos* transcription. Minami et al[23] have shown that Lck, a member of the Src-family, becomes activated by the stimulation with IL-2, and this PTK activation correlates well with the accumulation of c-*fos* transcripts. On the other hand, expression of a dominant negative form of Jak2 or Jak3 was demonstrated to suppress the c-*fos* transcription induced by granulocyte-macrophage CSF (GM-CSF)[24] or IL-2,[25] respectively. Although these data place Src-family and Jak-family members in the c-*fos* regulation pathways, little is still understood for the molecular mechanism by which they activate the c-*fos* promoter. We and others have already shown that Lyn, a member of the Src-family, can phosphorylate and activate Tec and Btk in cells.[26,27] Thus, the Tec-family members are likely to work downstream of the Src-family kinases in vivo. Therefore, it would be an intriguing question whether Tec is directly involved in the regulation of the c-*fos* promoter activity in the hematopoietic system.

To address this issue, here we transiently introduced a reporter plasmid (p*fos*/luc), containing the promoter region of

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the c-*fos* gene and the luciferase cDNA, into mouse BA/F3-HGMRαβ cells[28] which express the high-affinity receptors for human GM-CSF. pSRα plasmids carrying the cDNAs of various nonreceptor PTKs were co-introduced to compare the ability of each PTK to modulate c-*fos* promoter activity. Interestingly, we could observe that Tec was one of the most potent PTKs in the ability of the reporter gene activation. On the contrary, introduction of a kinase-deleted Tec could suppress the cytokine-driven c-*fos* activation in a dose-dependent manner. Because Jak2 expression also activated the c-*fos* promoter in our assay, we next investigated the functional and physical interaction between Tec and Jak2 in the context of c-*fos* activation mechanism. Co-expression of dnJak2 could block the Tec-driven c-*fos* activation, suggesting that Jak2 may work at a point downstream of Tec. Surprisingly, in both 293 cells and insect cells we could show that Tec and Jak2 can not only associate with, but also phosphorylate, each other. Our data indicate that the Src-, Tec-, and Jak-family members functionally interact to transduce the cytokine-driven c-*fos* activation mechanism.

MATERIALS AND METHODS

Cell lines. BA/F3 cells[29] were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and 25 U/mL of mouse IL-3. The BA/F3-hGMRαβ cells and a human GM-CSF–dependent cell line, UT-7,[30] were maintained in the same medium with 10% FCS and 1 ng/mL of human GM-CSF. 293 cells (American Type Culture Collection [ATCC], Rockville, MD) were maintained in Dulbecco's modified Eagle medium/F12 (DMEM/F12; GIBCO-BRL) containing 10% FCS and 2 mmol/L L-glutamine. Sf21 cells (Invitrogen, San Diego, CA) were grown in suspension at 28°C in the SF-900 II serum-free medium (GIBCO-BRL) without CO_2 supply. For the stimulation experiments, UT-7 cells were cultured in the starvation medium (RPMI 1640 medium with 0.5% FCS, 100 μg/mL transferrin [Boehringer Mannheim, Mannheim, Germany] and 100 μg/mL bovine serum albumin [Boehringer Mannheim]) at the concentration of 5×10^5 cells/mL for 12 hours, then at the concentration of 1×10^7 cells/mL in the same medium for 0.5 hour. The cells were stimulated with 10 ng/mL of human GM-CSF for the period of 5 minutes unless otherwise indicated.

Immunoprecipitation and in vitro kinase assay. The cDNA of mouse Tec type IV,[12] mouse Jak2,[31] dominant negative Jak2,[24] mouse Lyn A,[32] Syk with an N-terminal gp120 epitope tag,[33] or dominant negative Ras was ligated with the pSRα expression vector to generate pSRα-Tec, pSRα-Jak2, pSRα-dnJak2, pSRα-Lyn, pSRα-Syk, or pSRα-dnRas, respectively. To construct the cDNA encoding a kinase-deleted Tec (TecΔKD), Tec cDNA was digested by *Bpu*1102I, blunt-ended by T4 DNA polymerase, and the 3'-fragment encoding the kinase domain was removed. Introduction of the expression plasmids into 293 cells was performed by the calcium phosphate method. UT-7 or 293 cells were rinsed once with ice-cold phosphate-buffered saline (PBS) supplemented with 0.1 mmol/L Na_3VO_4, and resuspended into the 1%-lysis buffer (1% Nonidet P-40, 50 mmol/L Tris-HCl, 7.4, 150 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 200 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). After incubation on ice for 30 minutes, cell extracts were centrifuged to remove insoluble materials. Tec or Jak2 was immunoprecipitated from 1.5 to 2 mg of the cell lysates by anti-Tec serum[12] or anti-Jak2 sera (Santa Cruz Biotechnology, Santa Cruz, CA and Upstate Biotechnology, Lake Placid, NY), respectively, and was eluted into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Where indicated, cells were solubilized by the 0.1%-lysis buffer containing 0.1% of NP-40 instead of 1%.

For the in vitro kinase assay, the immune complexes were washed three times with the 1%-lysis buffer, three times with the kinase buffer (20 mmol/L Tris-HCl, 7.4, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$), and finally incubated with 0.37 MBq of [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) for 15 minutes at 30°C. For the assay of Jak2 activity, a synthetic substrate of Jak2 (Upstate Biotechnology) was added to the reaction (20 μg/experiment). Samples of the Jak2 kinase assay were subjected to Tricine-SDS-PAGE.

Immunoblotting. Total cell lysates (10 μg/lane) and the immune complexes were separated through 7.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA). The membranes were incubated for 1 hour at room temperature in TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) with 4% bovine serum albumin (Fraction V; Sigma, St Louis, MO). The membranes were then incubated with anti-Tec serum (1:10,000 dilution), anti-Jak2 serum, anti-Lyn serum,[26] anti-gp120 epitope tag antibody (H902), or anti-phosphotyrosine antibody (4G10; Upstate Biotechnology) for 1 hour at room temperature in TBST. Specific bindings of the antibodies were visualized by the ECL detection system (Amersham) according to the manufacturer's instructions.

Metabolic labeling and phosphoamino acid analysis. UT-7 cells were cultured at the concentration of 1×10^7 cells/mL in phosphate-free RPMI 1640 medium (GIBCO-BRL) supplemented with 5% dialyzed FCS (GIBCO-BRL) and 37 MBq/mL of [^{32}P]orthophosphate for 1 hour, and then stimulated with human GM-CSF for 5 minutes. Tec was immunoprecipitated from the cells, blotted onto a PVDF membrane, and incubated in 1 N KOH at 55°C according to the method of Kamps and Sefton.[34] Phosphoamino acid contents of pp70^{Tec} were determined as described earlier.[12]

Luciferase reporter assay. With the c-*fos* promoter-luciferase plasmid (p*fos*/luc) as a reporter, the expression plasmid of each kinase was introduced into BA/F3-hGMRαβ cells by electroporation according to the method of Watanabe et al[35] with minor modifications. Briefly, 1×10^7 of BA/F3-hGMRαβ cells were resuspended into 200 μL of OPTI-MEM I medium (GIBCO-BRL) and mixed with the expression vector DNAs (5 μg per construct unless otherwise indicated) plus the p*fos*/luc reporter plasmid (2 μg). Total amounts of plasmid DNAs in each set of electroporation were adjusted to be equal by adding the appropriate amounts of the blank vector DNA. After electroporation with the GenePulser apparatus (BioRad, Hercules, CA) at the condition of 200 V and 960 μF, cells were resuspended into 30 mL of RPMI 1640 medium with 10% FCS and cultured for 5 hours. The samples were further cultured for 5 hours either unstimulated or stimulated with 25 U/mL of mouse IL-3 or 5 ng/mL of human GM-CSF. The luciferase activities were measured by using the Luciferase Assay System (Promega, Madison, WI), and are shown as relative light units/min/μg of protein. The Elk activity was assayed in BA/F3 cells by using the PathDetect in vivo reporting system (Stratagene, La Jolla, CA). The MEK1 inhibitor (PD98059; New England Biolabs, Beverly, MA) was dissolved in dimethyl sulfoxide (DMSO) and add to the culture at the concentration of 50 μmol/L. The p*fos*/luc mutants were constructed by inserting the mutant promoter fragments[36] into pGL3-Basic plasmid (Promega).

Recombinant baculoviruses. The cDNAs of Tec and Jak2 were inserted into the pFastBacHT and pFastBac1 plasmids (both from GIBCO-BRL), respectively. The recombinant baculoviruses based on these plasmids were generated by the Bac-to-Bac baculovirus expression systems (GIBCO-BRL), and were used to infect Sf21 cells at the multiplicity of infection (MOI) of 1.0. After 48 to 72 hours of culture, cells were harvested and lysed as described above.

RESULTS

Tec is involved in the signaling pathway of GM-CSF receptor. To investigate whether Tec is involved in the signaling mechanism mediated by GM-CSF receptor (GMR), Tec was immunoprecipitated from a human GM-CSF–dependent cell line, UT-7, with or without the GM-CSF stimulation, and was immunoblotted with anti-phosphotyrosine antibody (αP-Tyr Ab). As shown in the upper panel of Fig 1A, GM-CSF stimulation of UT-7 cells for 5 minutes could clearly induce tyrosine-phosphorylation of Tec (indicated by an arrow) and a Tec-associated p56. The identity of this p56 is yet to be determined although we confirmed that $p52^{shc}$ and $p56^{lyn}$, both of which are known to be associated with Tec, have the same electrophoretic mobility with that of the "p56." The same membrane was reblotted with anti-Tec serum to prove that equivalent amounts of Tec were precipitated (lower panel). We could not detect a significant level of Btk expression in UT-7 cells by using an anti-Btk antibody (M-138; Santa Cruz Biotechnology). We next examined the time course of Tec phosphorylation. Tec was immunoprecipitated from UT7 cells with various periods of GM-CSF stimulation, and probed with αP-Tyr Ab. As shown in Fig 1B, tyrosine-phosphorylation of Tec was induced as rapidly as 1 minute after the stimulation, reached to the maximum level in 5 to 10 minutes, and decreased thereafter. Thus, the phosphorylation of Tec in response to GM-CSF is rapid and transient. To examine whether the kinase activity of Tec is also affected in response to GM-CSF, Tec was immunoprecipitated from UT-7 cells with or without GM-CSF stimulation and subjected to an in vitro kinase assay without exogenous substrates. As shown in Fig 1C and D, stimulation with GM-CSF for 5 minutes could enhance the auto-phosphorylation activity of Tec.

To directly estimate the phosphotyrosine contents, Tec was immunoprecipitated from UT-7 cells metabolically labeled with [^{32}P]orthophosphate, separated through 7.5% SDS-PAGE, blotted onto a PVDF membrane, and incubated in 1 N KOH to enrich the signals of phosphotyrosine. Autoradiography of the membrane could show that GM-CSF can induce phosphorylation of $pp70^{Tec}$ (Fig 1E). The phosphoamino acid contents of this $pp70^{Tec}$ were then examined by thin-layer chromatography, showing that phosphorylation of tyrosine residues was actually induced by the stimulation with GM-CSF (Fig 1F). These data imply that Tec is involved in the intracellular signaling mechanism mediated by GMR.

Tec is involved in cytokine-driven activation of c-fos *transcription.* To examine whether Tec mediates cytokine-driven activation of the c-*fos* gene, the p*fos*/luc plasmid in which the luciferase expression is controlled by the c-*fos* promoter was transfected into BA/F3-hGMRαβ cells by electroporation together with the pSRα-based expression plasmid of Syk, Lyn, Jak2, or Tec. As shown in Fig 2A, stimulation of the vector-transfected BA/F3-hGMRαβ cells with either GM-CSF or IL-3 could enhance the luciferase reporter activity. Co-introduction of the Syk kinase with an N-terminal tag[33] did not affect the luciferase activity, suggesting Syk is not involved in the c-*fos* activation mechanism in BA/F3 cells. In contrast, introduction of Lyn kinase significantly elevated the luciferase activity of the unstimulated basal level. However, cytokine stimulation of the cells could not further enhance the reporter activity. This lack of cytokine-responsiveness in Lyn-transfected cells was confirmed in repeated experiments. As previously reported, introduction of Jak2 could elevate the reporter activity of the unstimulated state as well as of cytokine-stimulated states. Interestingly, Tec introduction elevated the reporter activity of the unstimulated state similar to the level obtained by the Lyn-transfection. In contrast to the case of Lyn, Tec expression could also strongly enhance the reporter activity in response to GM-CSF or IL-3. Appropriate expression of each kinase was confirmed by the immunoblot analysis of the total cell lysates (Fig 2B).

We then directly tested whether Tec is an intermediate in the cytokine-driven c-*fos* activation pathway by using a kinase-deleted Tec (TecΔKD). As shown in Fig 2C, introduction of pSRα-TecΔKD into BA/F3-hGMRαβ cells suppressed the c-*fos* promoter activity stimulated by GM-CSF or IL-3 in a dose-dependent manner. These data strongly support the idea that Tec directly mediates the cytokine-driven c-*fos* activation. It is widely known that c-*fos* transcription is regulated via the Ras-MAPK pathway. Therefore, we checked whether the Tec-driven c-*fos* activation is transduced through Ras by coexpressing a dominant negative form of Ras (dnRas). As shown in Fig 2D, coexpression of dnRas could totally block the Tec-driven activation of the c-*fos* gene. Thus, Tec is likely to drive the c-*fos* activation through a Ras-regulated mechanism. By using the PathDetect in vivo reporting system (Stratagene), we then asked whether Elk, a transcriptional factor acting downstream of Ras, is involved in the Tec-mediated c-*fos* activation. The pFA-Elk plasmid, encoding the fusion protein consisting of the DNA binding domain of yeast GAL4 and the activation domain of Elk, was transfected into BA/F3 cells together with Tec-expression plasmids and the reporter pFR-luc plasmid in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites (Fig 2E). In the pSRα-transfected cells ("V" part), IL-3 stimulation resulted in the elevation of luciferase activity, which suggests that Elk is activated in response to IL-3. Introduction of Tec markedly increased the reporter activity both in the unstimulated and stimulated states ("Tec" part). In contrast, transfection of TecΔKD suppressed the luciferase activity, indicating that Elk-pathway is involved in the Tec-driven c-*fos* activation process. We also tested whether MEK1, an intermediate between Ras and Elk, plays a role in this c-*fos* activation mechanism. After electroporation with pFA-Elk and pFR-luc, BA/F3 cells were cultured for 4 hours without IL-3, and then for 1 hour with an MEK1 inhibitor, PD98059, before the IL-3 stimulation. As shown in Fig 2F, treatment with PD98059 significantly suppressed the Tec-driven Elk-activation. In a separate line of experiment, we investigated what kind of transcriptional factor(s) is responsible for the Tec-mediated c-*fos* transcription. The c-*fos* promoter fragment is known to contain four *cis*-regulatory elements, namely, the *sis*-inducible element (SIE), the serum response element (SRE), the c-*fos* AP-1 binding element (FAP), and the calcium and cyclic AMP response element (Ca/CRE) (Fig 2G). These regulatory sequences are presumed to work in concert to control the c-*fos* transcription in a tissue- and stimulus-specific fashion.[36] By using the p*fos*/luc mutants in which point mutations were

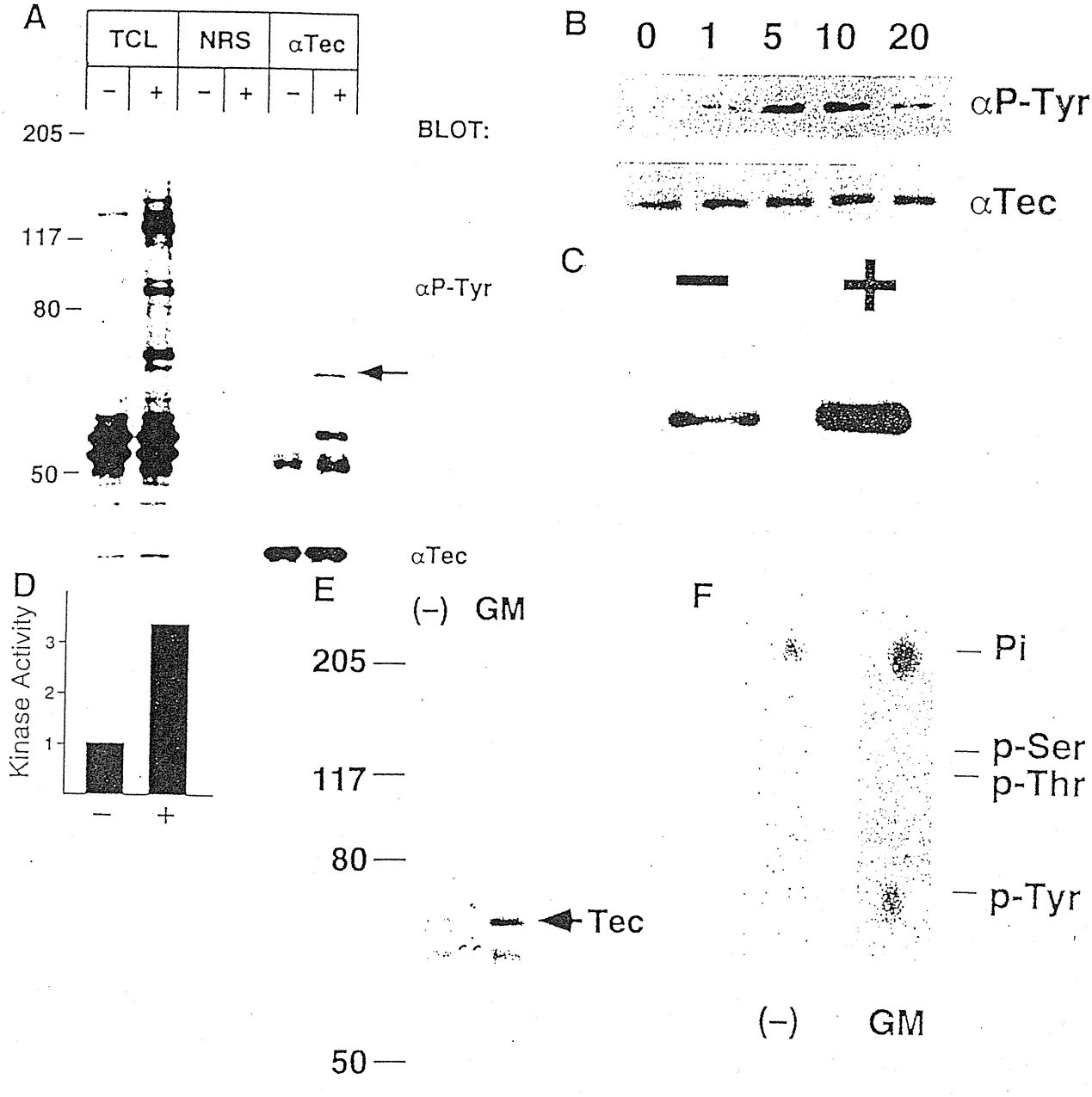

Fig 1. Tec is involved in the signaling pathway of GM-CSF receptor. (A) UT-7 cells (1×10^7) were cultured in the starvation medium for 12 hours and then stimulated with 10 ng/mL of human GM-CSF (+) for 5 minutes or left unstimulated (−). Tec was immunoprecipitated from each fraction (αTec), subjected to 7.5% SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (αP-Tyr). Total cell lysates (TCL; 10 μg/lane) and the immunoprecipitates by normal rabbit serum (NRS) prepared from the same set of cells were also analyzed. The position of Tec is indicated by an arrow. The molecular weight standards ($\times 10^{-3}$) are shown at the left. The same membrane was reblotted with anti-Tec serum to show the amounts of Tec precipitated (lower panel). (B) UT-7 cells were stimulated with GM-CSF (10 ng/mL) for 0, 1, 5, 10, or 20 minutes as indicated at the top. Tec was immunoprecipitated from each fraction (1×10^7 cells), and was immunoblotted with anti-phosphotyrosine antibody (αP-Tyr) or anti-Tec serum (αTec). (C) Tec was immunoprecipitated from 1×10^7 of UT-7 cells with (+) or without (−) 5 minutes of GM-CSF stimulation, and was subjected to an in vitro kinase assay. Autophosphorylation of $pp70^{Tec}$ is shown. (D) Specific kinase activity of the Tec protein (^{32}P-incorporation/protein amount) with (+) or without (−) the GM-CSF stimulation was calculated by densitometric analysis and shown as arbitrary units. (E) Tec was immunoprecipitated from UT-7 cells (1×10^7), with (GM) or without (−) the GM-CSF stimulation (10 ng/mL), metabolically labeled with [^{32}P]orthophosphate (37 MBq/mL), and was analyzed by 7.5% SDS-PAGE. The proteins were blotted onto a PVDF membrane, and heated in 1 N KOH to decrease the backgrounds of serine- and threonine-phosphorylation. The position of Tec is indicated. (F) $pp70^{Tec}$ in (E) was subjected to the phosphoamino acid analysis. The positions of free phosphate (Pi), phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) are indicated at the right.

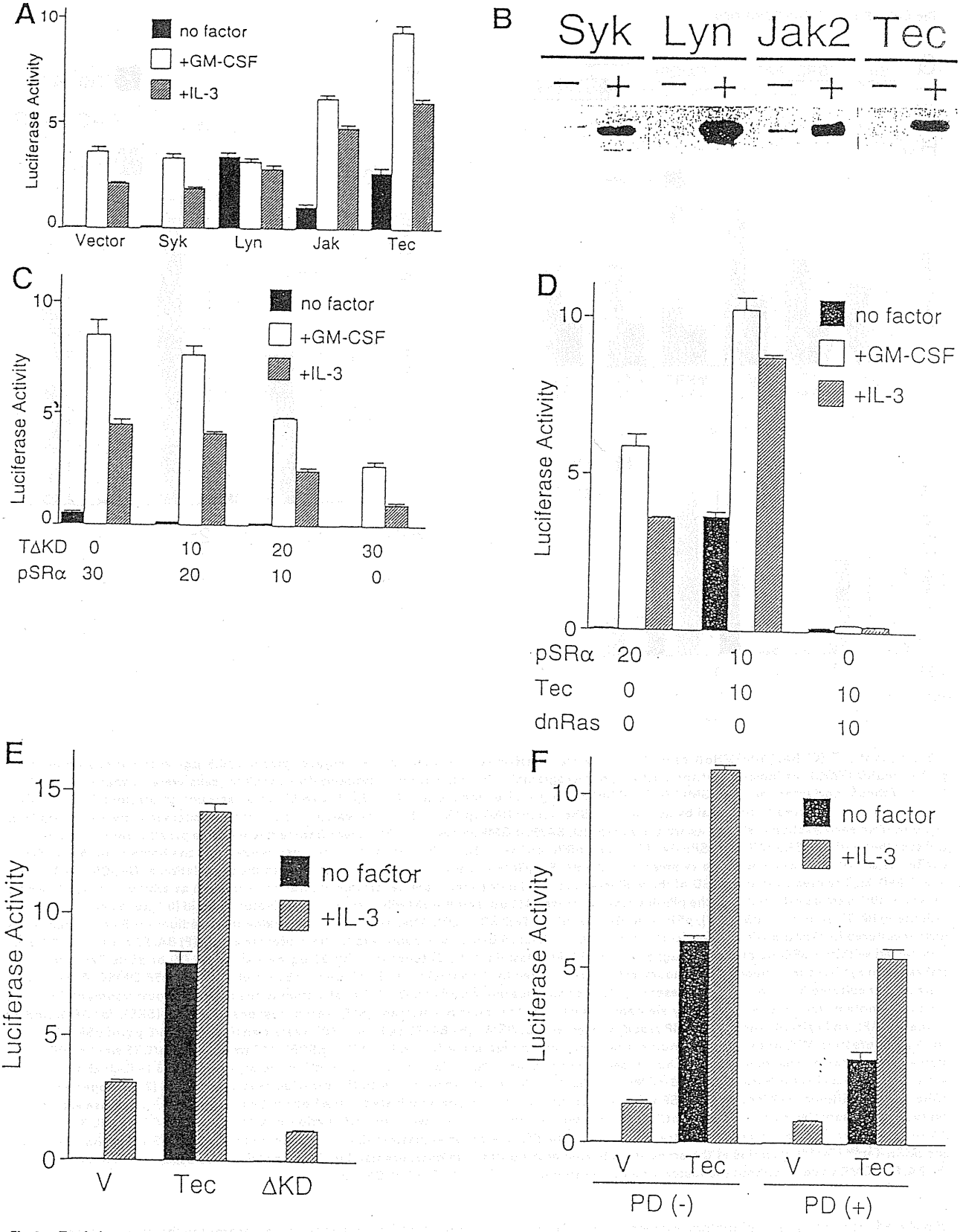

Fig 2. Tec is involved in the cytokine-driven activation of c-*fos* proto-oncogene. (A) BA/F3-hGMRαβ cells (1×10^7) were transfected with the p*fos*/luc reporter plasmid (2 μg) together with 5 μg each of the pSRα (Vector), pSRα-Syk (Syk), pSRα-Lyn (Lyn), pSRα-Jak2 (Jak), or pSRα-Tec (Tec). After 5 hours of incubation in cytokine-free medium, the cells were further cultured for 5 hours without (no factor) or with 5 ng/mL of human GM-CSF (+GM-CSF) or 25 U/mL of mouse IL-3 (+IL-3). Luciferase activity was assayed in each fraction and calculated as relative light units (RLU)/min/μg of protein. The mean value plus SD of the luciferase activities in triplicate samples from each fraction is shown as arbitrary units. (B) BA/F3-hGMRαβ cells were transfected with pSRα-Syk, pSRα-Lyn, pSRα-Jak2, or pSRα-Tec, and cultured for 24 hours in the presence of IL-3. Total cell lysates (10 μg/lane) were prepared from each set (+) and untransfected BA/F3-hGMRαβ cells (−), and were immunoblotted with the antibodies against the corresponding kinases.

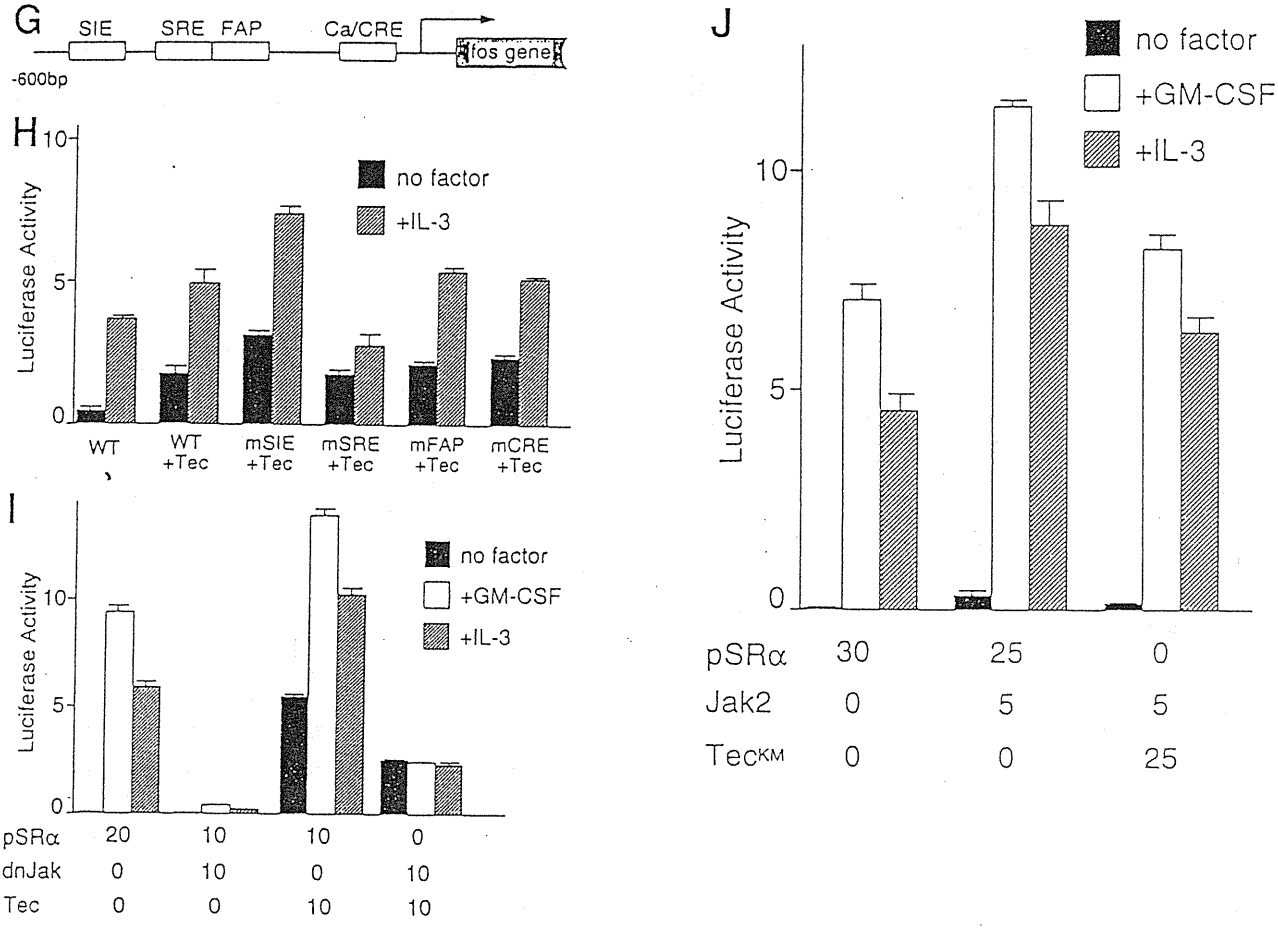

Fig 2 (Cont'd). (C) BA/F3-hGMRαβ cells (1 × 10^7) were transfected with the p*fos*/luc reporter plasmid (0.5 μg) with various amounts of pSRα-TecΔKD (TΔKD) as indicated at the bottom (in micrograms). After starvation in cytokine-free medium, cells were further cultured for 3 hours without (no factor) or with GM-CSF (+GM-CSF; 5 ng/mL) or mouse IL-3 (+IL-3; 25 U/mL). Total amount of plasmid DNA for each transfection was adjusted to be equal by adding the pSRα vector DNA (pSRα). The mean value plus SD of the luciferase activities in triplicate samples from each fraction is shown as arbitrary units. (D) BA/F3-hGMRαβ cells (1 × 10^7) were transfected with the p*fos*/luc reporter plasmid (2 μg) together with of pSRα (pSRα), pSRα-Tec (Tec), and pSRα-dnRas (dnRas) plasmids at the amounts indicated at the bottom (in micrograms). Luciferase activity was assayed in the samples from the BA/F3-hGMRαβ cells without (no factor) or with the stimulation of GM-CSF (+GM-CSF) or IL-3 (+IL-3). The mean value plus SD of the luciferase activities in triplicate samples from each fraction is shown as arbitrary units. (E) BA/F3 cells (1 × 10^7) were transfected with the pFR-luc reporter plasmid (1 μg) and the pFA-Elk fusion transactivator plasmid (0.1 μg) by electroporation together with 30 μg each of pSRα (V), pSRα-Tec (Tec), or pSRα-TecΔKD (ΔKD). After starvation in cytokine-free medium for 5 hours, cells were further cultured for 3 hours without (no factor) or with IL-3 (+IL-3; 25 U/mL), and subjected to the luciferase assay. (F) BA/F3 cells (1 × 10^7) were transfected with the pFR-luc plasmid (1 μg) plus the pFA-Elk plasmid (0.1 μg) together with 30 μg each of pSRα (V) or pSRα-Tec (Tec). After starvation in cytokine-free medium for 4 hours, cells were cultured for 1 hour with DMSO [PD(−)] or 50 μmol/L of PD98059/DMSO [PD(+)]. Cells were further cultured for 3 hours in the presence (+IL-3) or absence (no factor) of IL-3.(G) The structure of the c-*fos* promoter fragment. The c-*fos* promoter contains four known regulatory elements, namely, the *sis*-inducible element (SIE), serum response element (SRE), *fos* AP-1 binding element (FAP), and calcium and cyclic AMP response element (Ca/CRE). (H) BA/F3 cells (1 × 10^7) were transfected with 5 μg of pSRα-Tec (Tec) and 2 μg of p*fos*/luc (WT) or the p*fos*/luc mutants carrying point mutations at SIE (mSIE), SRE (mSRE), FAP (mFAP), or Ca/CRE site (mCRE). After starvation in cytokine-free medium for 5 hours, cells were further cultured for 3 hours without (no factor) or with IL-3 (+IL-3; 25 U/mL), and subjected to the luciferase assay. (I) BA/F3-hGMRαβ cells (1 × 10^7) were transfected with the p*fos*/luc reporter plasmid (2 μg) together with of pSRα (pSRα), pSRα-dnJak2 (dnJak) and pSRα-Tec (Tec) plasmids at the amounts indicated at the bottom (in micrograms). Luciferase activity was assayed in the samples from the BA/F3-hGMRαβ cells without (no factor) or with the stimulation of GM-CSF (+GM-CSF) or IL-3 (+IL-3). (J) BA/F3-hGMRαβ cells (1 × 10^7 cells) were transfected with the p*fos*/luc reporter plasmid (2 μg) together with of pSRα (pSRα), pSRα-Jak2 (Jak2) and pSRα-Tec^{KM} (Tec^{KM}) plasmids at the amounts indicated at the bottom (in micrograms). Luciferase activity was assayed in the samples from the BA/F3-hGMRαβ cells without (no factor) or with the stimulation of GM-CSF (+GM-CSF) or IL-3 (+IL-3).

introduced into individual regulatory elements (kind gifts of T. Curran), we here analyzed how each element contributes to the Tec-driven c-*fos* activation (Fig 2H). In accordance with the results in Fig 2E, mutations at SRE, the binding site of the Elk/TCF complex, significantly decreased the Tec-driven activation of c-*fos* transcription. These lines of evidence support the idea that Tec activates c-*fos* promoter through, at least in part, the Ras-MEK1-Elk pathway.

Because both of the Tec and Jak2 kinases could enhance cytokine-driven c-*fos* activation, we then tried to clarify whether Tec and Jak2 work in the same pathway or in a parallel manner to drive the c-*fos* promoter. First, Tec was introduced into

BA/F3-hGMRαβ cells with or without dominant negative Jak2 (dnJak2) to examine whether Jak2 is involved in the Tec-driven pathway (Fig 2I). Interestingly, expression of dnJak2 could suppress the cytokine-driven as well as Tec-driven luciferase activity, indicating that Jak2 acts downstream of Tec in the c-*fos* activation mechanism. We have also tested the possibility of the Tec-Jak2 interaction in the reverse direction. As shown in Fig 2J, introduction of a kinase-dead Tec^{KM} (Lys-397 at the ATP-binding site is replaced with Met) could slightly suppress the Jak2-driven activation of the c-*fos* gene. Although we could reproducibly observe this weak suppression (about 20% reduction), we do not yet have a strong proof that Tec is involved in a part of the Jak2-driven mechanism in the c-*fos* regulation.

Tec can phosphorylate Jak2 in cells. To understand how Tec and Jak2 can functionally interact with each other, we first examined the possibility that the former directly phosphorylates the latter. A kinase-dead Jak2 ($Jak2^{KE}$: Lys-882 in the ATP-binding site is replaced with Glu) was expressed in 293 cells with or without Tec, immunoprecipitated by anti-Jak2 serum and blotted with αP-Tyr Ab. To our surprise, as shown in the upper panel of Fig 3A, $Jak2^{KE}$ could be phosphorylated by Tec in intact cells. Interestingly, the tyrosine-phosphorylated $p70^{Tec}$ was also identified in the anti-Jak2 immunoprecipitate, suggesting the physical interaction between the two PTKs. The same membrane was reprobed with anti-Jak2 serum to prove that equivalent amounts of Jak2 were precipitated (lower panel). This Tec phosphorylation of $Jak2^{KE}$ is not likely to arise from a nonspecific reaction by over-expressed Tec proteins, because Syk could not phosphorylate $Jak2^{KE}$ in a similar experiment in 293 cells (data not shown). We also examined the ability of Tec to phosphorylate Jak2 in the insect cell system. Sf21 cells derived from *Spodoptera frugiperda* were infected with the recombinant baculovirus expressing $Jak2^{KE}$ alone or in combination with the Tec-expressing or Tec^{KM}-expressing virus. After 2 days of incubation, $Jak2^{KE}$ was immunoprecipitated from the cells and probed with αP-Tyr Ab (upper panel of Fig 3B). As expected, phosphorylation of $Jak2^{KE}$ could be identified only when $Jak2^{KE}$ was coexpressed with kinase-active Tec. In contrast, coexpression of Tec^{KM} did not confer detectable tyrosine-phosphorylation on $Jak2^{KE}$. The same membrane was reblotted with anti-Jak2 serum to estimate the amounts of Jak2 immunoprecipitated. These data favor the idea that Jak2 is a direct substrate of Tec in vivo. In these experiments, Jak2-phosphorylation by Tec was clearly and reproducibly observed when we used, for immunoprecipitation, anti-Jak2 serum against the C-terminal tail of Jak2 (C-20; Santa Cruz Biotechnology), not the one against amino acid positions 758-776 (Upstate Biotechnology), which may imply that the target site(s) of Tec is localized within or very close to the 758-776 region.

We then tested whether this trans-phosphorylation of Jak2 by Tec affects the kinase activity of the Jak2 protein. Jak2 was expressed in Sf21 cells with or without Tec, immunoprecipitated by anti-Jak2 serum, and was subjected to an in vitro kinase assay with a synthetic substrate peptide. As shown in Fig 3C, coexpression of Tec did not affect the phosphorylation of the Jak2-substrate. Because the immunoprecipitated $Jak2^{KE}$ could not phosphorylate the peptide at all (lane "J^E + T"), phosphorylation of the peptide in the other lanes was supposed to be carried out by Jak2, not by the coprecipitated kinases from Sf21 cells. We observed that Jak2 was expressed in equal amounts in each Sf21 fraction, as judged from the immunoblotting of the total cell lysates with anti-Jak2 serum (data not shown). Similar results of the in vitro kinase assay were obtained with Jak2 expressed in 293 cells (data not shown). Phosphorylation of Jak2 without the modulation of its activity may be used in vivo for collecting signaling molecules to Jak2 protein. We checked this possibility by using the BA/F3 cells expressing an SH3-deleted active Tec (TecΔSH3).[37] Jak2 was immunoprecipitated from parental BA/F3 cells and two BA/F3 transfectants stably expressing TecΔSH3, and blotted with αP-Tyr Ab. As shown in Fig 3D, many tyrosine-phosphorylated proteins become associated with Jak2 only when TecΔSH3 is coexpressed, which may indirectly support the possibility above.

Jak2 can phosphorylate Tec at Tyr-518. To investigate the phosphorylation reaction in the reverse direction between the two kinases, Tec^{KM} was introduced into 293 cells with or without Jak2, and was analyzed for tyrosine-phosphorylation (upper panel, Fig 4A). Because it was already known that Tec can be directly phosphorylated and activated by Lyn PTK, a coexpression experiment of Lyn kinase was used as a positive control. To our surprise again, Tec could be in vivo phosphorylated by Jak2 as well as by Lyn. The same membrane was then probed with anti-Tec serum to estimate the amounts of Tec precipitated (lower panel). Therefore, Tec and Jak2 can trans-phosphorylate each other.

We then tried to map the phosphorylation site of Tec by Jak2. Yamashita et al[37] previously demonstrated that the deletion of the internal SH3 domain results in hyperphosphorylation and activation of Tec in vivo. In addition, Tec kinase has a tentative autophosphorylation site (Tyr-518) in the activation loop of its catalytic domain, corresponding to Tyr-416 in c-Src. Therefore, we investigated the possibility that either of the SH3 domain or Tyr-518 is the target site of Jak2 and Lyn kinases. Tec^{KM}, Tec^{KM}ΔSH3 (the SH3 domain of Tec^{KM} is deleted), or $Tec^{KM.YF}$ (Tyr-518 of Tec^{KM} is replaced with Phe) was expressed in 293 cells either alone or in combination with Jak2 or Lyn. Tec was then immunoprecipitated and probed with αP-Tyr Ab. As shown in the upper panel of Fig 4B, internal deletion of the SH3 domain did not decrease the phosphorylation of Tec^{KM} by Jak2. On the other hand, the Phe-substitution for Tyr-518 nearly completely abolished the phosphorylation of Tec^{KM} protein ("$T^{M.518F}$" part). Hence, Tyr-518 of Tec is the target site of both Jak2 and Lyn. The same membrane was reblotted with anti-Tec serum to prove that equivalent amounts of Tec proteins were immunoprecipitated (lower panel). It is not likely that Jak2 phosphorylated Tec indirectly through Lyn (or other Src-family kinases), because the kinase activity of Lyn was not affected by the coexpression of Jak2 when transiently expressed in 293 cells (data not shown). The amino acid sequences surrounding this Tyr-518 position in Tec-family kinases are well conserved (Fig 4C). Therefore, it would not be surprising if other members of the Tec-family are also controlled by Src- and Jak-family kinases through a similar phosphorylation mechanism.

Because Jak2 and Lyn can phosphorylate the same residue (Tyr-518) of Tec, we speculated that Jak2 may activate Tec as in the case of Lyn. Tec or Tec^{KM} was expressed in 293 cells either alone or in combination with Jak2. Tec was immunoprecipitated from each set and subjected to an in vitro kinase assay to test its

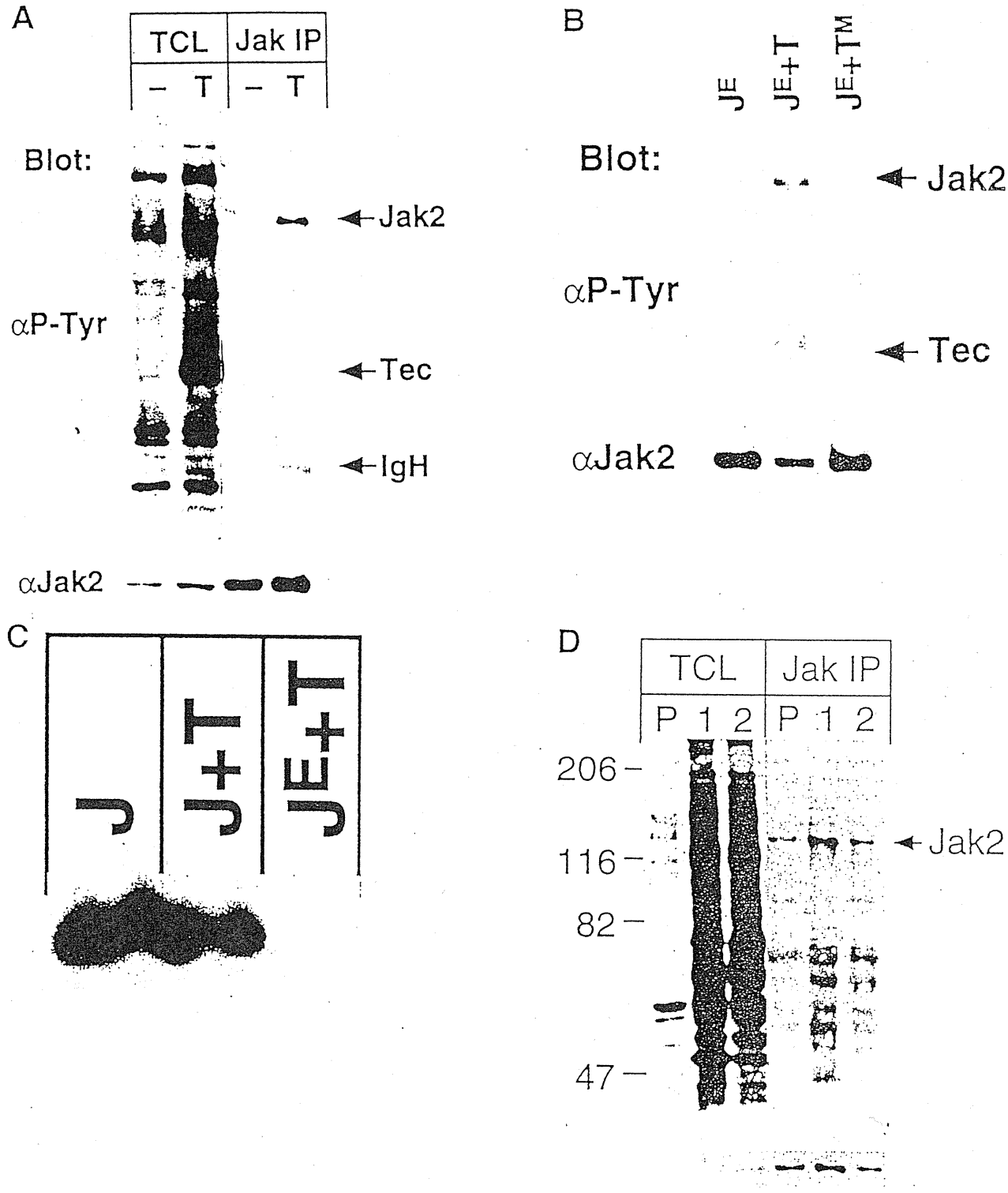

Fig 3. Tec can phosphorylate Jak2 in both mammalian and insect cells. (A) Jak2^{KE} was immunoprecipitated from 2 × 10^6 of 293 cells expressing Jak2^{KE} with (T) or without (–) Tec. Total cell lysates (TCL, 10 μg/lane) and anti-Jak2 immunoprecipitates (Jak IP) were electrophoresed and probed with anti-phosphotyrosine antibody (αP-Tyr) or anti-Jak2 serum (αJak2). The positions of Jak2 (Jak2), Tec (Tec), and the Ig heavy chain (IgH) are indicated at the right. (B) Jak2^{KE} was immunoprecipitated from Sf21 cells infected with Jak2^{KE} expressing baculovirus (J^E) alone or in combination with Tec-expressing (T) or Tec^{KM}-expressing (T^M) virus. The immunoprecipitates were separated through 7.5% SDS-PAGE and probed with anti-phosphotyrosine antibody (αP-Tyr) or anti-Jak2 serum (αJak2). The positions of Jak2 (Jak2) and Tec (Tec) are indicated at the right. (C) Jak2 was immunoprecipitated from Sf21 cells expressing Jak2 (J) or Jak2^{KE} (J^E) either alone or in combination with Tec (T). The immunoprecipitates were incubated with [γ-^{32}P]ATP and the synthetic Jak2-substrate, and subjected to Tricine-SDS-PAGE. Phosphorylation of the Jak2-substrate is shown. (D) Total cell lysates (TCL: 10 μg/lane) and the anti-Jak2 immunoprecipitates (Jak IP) were prepared from parental BA/F3 cells (P) and two BA/F3 clones (1 and 2) stably expressing TecΔSH3, and immunoblotted with αP-Tyr Ab (upper panel) or anti-Jak2 serum (lower panel). The position of Jak2 is indicated at the right. The positions of molecular weight standards (×10^{-3}) are also shown at the left.

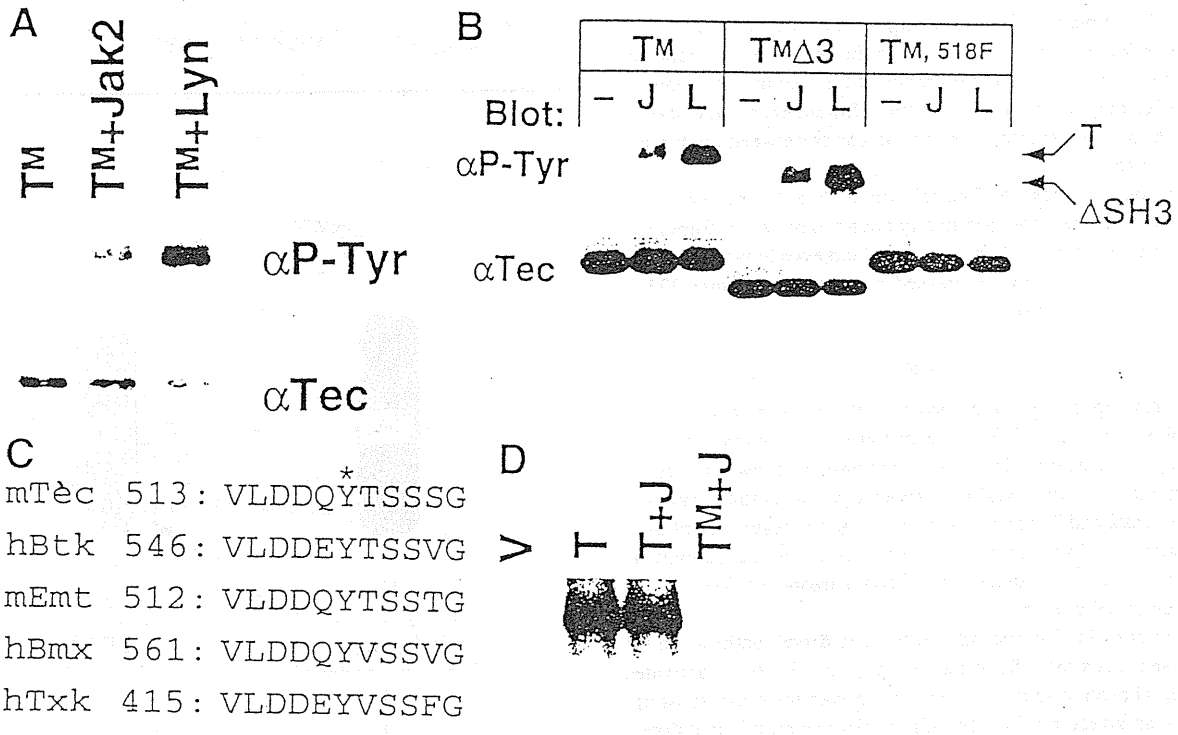

Fig 4. Jak2 can phosphorylate Tec at Tyr-518. (A) The kinase-dead Tec^{KM} (T^{M}) was expressed in 293 cells either alone or in combination with Jak2 (Jak2) or Lyn (Lyn) kinase. Tec was immunoprecipitated from each fraction, and probed with anti-phosphotyrosine antibody (αP-Tyr) or anti-Tec serum (αTec). (B) Tec^{KM} (T^{M}), Tec^{KM}ΔSH3 (T^{M}Δ3), or Tec^{KM,YF} (T^{M,518F}) was expressed in 293 cells either alone (–) or in combination with Jak2 (J) or Lyn (L). Tec was immunoprecipitated from each fraction, and blotted with anti-phosphotyrosine antibody (αP-Tyr) or anti-Tec serum (αTec). The positions of full-length Tec (T) and SH3-deleted (ΔSH3) forms are indicated at the right. (C) The amino acid sequences of the Tec-family kinases, surrounding the tyrosine residues corresponding to Tyr-518 in mouse Tec, are compared. The asterisk indicates the position of the phosphorylated tyrosine. At the left shown are the numbers of amino acid positions of mouse Tec,[2] human Btk,[10,11] mouse Emt/Itk/Tsk, [9,41,42] human Bmx,[43] and human Txk.[44] (D) pSRα (V), pSRα-Tec (T), or pSRα-Tec^{KM} (T^{M}) was transfected into 293 cells with or without pSRα-Jak2 (J). Tec was immunoprecipitated from each fraction, and incubated with [γ–^{32}P]ATP without exogenous substrates. Autophosphorylation of pp70^{Tec} in each sample is shown.

auto-phosphorylation activity. Unexpectedly, as shown in Fig 4D, autophosphorylation level of p70^{Tec} was not altered irrespective of the presence of Jak2 PTK. In contrast, coexpression of Lyn kinase could activate Tec as reported previously (data not shown). Thus, although Jak2 and Lyn can phosphorylate the same site of Tec, we observed only Lyn can activate the Tec kinase under the sensitivity of our assay.

Tec can bind to Jak2 in insect cells. We then tested whether Tec and Jak2 can physically associate with each other in cells. Recombinant baculovirus expressing Tec or Tec^{KM} was used to infect Sf21 cells either alone or in combination with the virus expressing Jak2 or Jak2^{KE} (Fig 5). Jak2 was immunoprecipitated from the cells lysed by the 0.1% lysis buffer, and immunoblotted with anti-Tec serum. Tec could be identified very weakly in the Jak2-immune complex, and more clearly found was Tec^{KM} in the Jak2-immune complex (top panel). Also, Jak2^{KE} was shown to associate with Tec irrespective of the Tec-activity. Thus, Tec can weakly bind to Jak2 in insect cells, and this interaction does not require the kinase activity of Tec and Jak2. It should be noted that co-introduction of kinase-

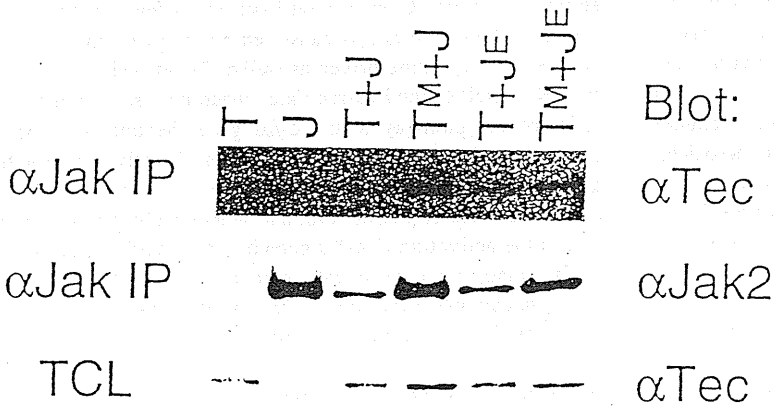

Fig 5. Tec can constitutively associate with Jak2 in Sf21 cells. Sf21 cells were infected with baculovirus expressing Tec (T), Jak2 (J), Tec^{KM} (T^{M}), and Jak2^{KE} (J^{E}) in the combinations indicated at the top. Jak2 was immunoprecipitated from each cells lysed by the 0.1%-lysis buffer (αJak IP), and probed with either anti-Tec serum (αTec) or anti-Jak2 serum (αJak2). Total cell lysates (TCL: 10 μg/lane) of each fraction were also probed with anti-Tec serum to estimate the expression level of Tec.

active Tec always reduced the expression level of Jak2 in Sf21 cells (middle panel, and also confirmed in other repeated experiments). Therefore, difference of the amounts of coprecipitated Tec may have arisen from the different expression level of Jak2 (compare the intensities of the bands between the top and middle panels).

We could not test the Tec/Jak2 interaction in the reverse direction, since Jak2 nonspecifically bound to protein A-Sepharose beads and glutathione Sepharose 4B (and even to nickel agarose beads) when Jak2 was expressed abundantly in either 293 or Sf21 cells (data not shown).

DISCUSSION

In this report we have shown that Tec is involved in the signaling pathway of GMR, especially in the c-*fos* activation machinery. Because Jak2 was previously shown to be an intermediate in the cytokine-driven c-*fos* activation pathway, both of Jak2 and Tec should play a role in the regulation of c-*fos* transcription. Furthermore, our data with dnJak2 support an intriguing idea to place Jak2 downstream of Tec in the mechanism of c-*fos* activation.

How does Jak2 participate in the Tec-driven pathway to the c-*fos* gene? A simple hypothesis is that Jak2 becomes activated via the phosphorylation by Tec, and drives the c-*fos* transcription as an effector of the Tec kinase. However, this is unlikely because coexpression of Tec could not affect the activity of Jak2 in either mammalian or insect cells. The second explanation is that Jak2 may be required to fully activate Tec through the phosphorylation of Tec protein by Jak2. This assumption is again unlikely because (1) coexpression of Jak2 could not activate Tec in either 293 cells or Sf21 cells, and (2) coexpression of dnJak2 with Tec in 293 cells did not suppress the kinase activity or tyrosine-phosphorylation of Tec (data not shown). Therefore, Jak2 may not be a direct second messenger of Tec, but should be required for the appropriate function of Tec-substrates ("Substrate X" in Fig 6). There are several possible scenarios for such interaction. Jak2 may be, for instance, prerequisite to recruit the substrates of Tec into the cytokine receptor complex. It is well known that cytokine receptors are the good substrates of Jak-family kinases both in vitro and in vivo, and that a variety of signaling molecules become associated with the receptors through the phosphotyrosine-SH2 domain (or phosphotyrosine-binding [PTB] domain) interaction.[38] Thus, it is possible that the second messengers for c-*fos* activation can become accessible to Tec through the phosphorylation of receptors by Jak2. Another explanation may be that Tec collects its substrates by phosphorylating Jak2 and thereby making it bound to the Tec-substrates. In this scenario, Jak2 plays as a "bridge" to connect Tec and its effector molecules. There would be, again, the other possibility that Jak2 is required to phosphorylate the Tec-substrates and to let them associated with Tec. To determine which interaction really takes place in vivo, we have to identify the "Substrate X" responsible for the c-*fos* activation, and we should also clarify the phosphorylation site(s) of Jak2 by the Tec kinase.

Analysis of various deletion mutants of human GMR common β chain (β_c) showed that a central area in the cytoplasmic region of β_c is necessary for cytokine-dependent Shc phosphorylation, activation of Ras, and induction of the c-*fos* gene.[35,39] In

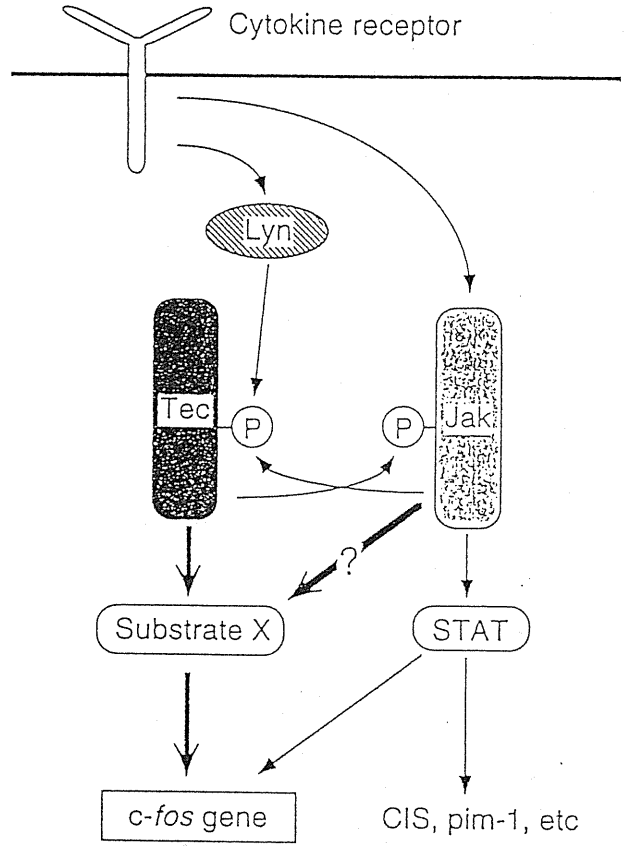

Fig 6. Cytokine-driven pathways to the c-*fos* proto-oncogene. When activated by Lyn, Tec phosphorylates "Substrate X" and triggers the signaling pathway linked to the c-*fos* activation. Tec and Jak2 can trans-phosphorylate each other. The biological significance of this phosphorylation in the context of c-*fos* activation mechanism is not settled yet. Jak2 is required for the appropriate function of "Substrate X" as well as for the phosphorylation of STATs. The STATs activation may also have some roles in the regulation of the c-*fos* transcription.

fibroblasts, Shc is already known to bind to Grb2 in a phosphorylation-dependent manner, and thereby to trigger the recruitment of SOS guanine nucleotide exchanging factor and the Ras activation.[40] Therefore, Shc/Ras may be a key component to drive the c-*fos* transcription also in blood cells. If this is the case, Shc would be an intriguing candidate for the "Substrate X" in Fig 6. Currently we have only a few data to support this hypothesis. First, because expression of dnRas could suppress the cytokine-driven as well as Tec-driven c-*fos* activation, Ras itself or the Ras-regulated machinery should be a relay point of the pathway to the c-*fos* gene. Second, we already proved that Shc can be associated (either directly or indirectly) with Tec in cells. However, it is yet to be shown whether Shc is a direct substrate of Tec in vivo, and whether Shc plays a central role in the activation of c-*fos* gene in the hematopoietic system.

Our mapping experiments evidenced that Tyr-518 is the major phosphorylation site of Tec by both Jak2 and Lyn. The fact that Jak2 is capable of phosphorylating Tec was also confirmed in another laboratory (T. Matsuda and J.N. Ihle, personal communication). However, only Lyn could enhance

the kinase activity of Tec in our experiments. Although we do not have any evidence to explain this discrepancy, several possibilities can be raised. First, as shown in Fig 4A and B, stoichiometry of Tec-phosphorylation was always higher when coexpressed with Lyn than with Jak2. Thus, low level of Tyr-518 phosphorylation by Jak2 may not be sufficient to demonstrate the enhancement of autophosphorylation activity in the anti-Tec immunoprecipitates. On the other hand, a weak but still significant tyrosine-phosphorylation of $Tec^{KM.518F}$ could be reproducibly observed in a longer exposure of the film, when coexpressed with either Lyn or Jak2 (data not shown). Thus, there may be additional phosphorylation sites by Jak2 and Lyn, and these minor sites may have a pivotal role in the regulation of Tec activity. It is also possible that Lyn and Jak2 bind to Tec at different sites, and that these bindings may render distinct allosteric effects on Tec molecules.

Our report has shown the presence of a "cross-talk" between Tec and Jak2 PTKs. Although Tec is the first PTK among non-Jak kinases shown capable of phosphorylating Jak2, it would not be surprising if the members of other PTK-families are also able to phosphorylate Jak kinases. Growth of blood cells would be controlled through these complexed networks among various PTKs, and our observation would be an important information to decipher the control mechanisms.

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