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Interleukin-6 Signals Activating *junB* and TIS11 Gene Transcription in a B-Cell Hybridoma

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The events in interleukin-6 (IL-6) signal transduction leading to primary response gene activation were analyzed in murine B-cell hybridoma and plasmacytoma cells which require IL-6 for growth. IL-6 stimulation of IL-6-deprived cells resulted in the rapid and transient tyrosine phosphorylation of a 160-kDa cellular protein (p160). This was followed by the highly selective induction of two primary response genes, *junB*/AP-1 transcription factor and TIS11. *junB* and TIS11 inductions were unaffected by cycloheximide, suggesting that posttranslational modifications accounted for their activation. Activation of *junB* and TIS11 transcription required rapid tyrosine kinase activity as well as a different protein kinase activity sensitive to the potent kinase inhibitor, H7, and activated following p160 tyrosine phosphorylation. This H7-sensitive kinase appears to be distinct from any well-characterized protein kinase-second messenger system. On the basis of these findings, we propose that IL-6-induced signal transduction proceeds through a novel protein kinase cascade which activates *junB* and TIS11 gene transcription.

Interleukin-6 (IL-6) is a pleiotropic cytokine regulating growth, differentiation, and other cellular functions in many cell lineages (reviewed in references 28 and 57). IL-6 functions as a growth factor for certain murine hybridomas and plasmacytomas (41, 56) as well as certain human myelomas (27). IL-6 stimulates the proliferation of mitogen-activated T cells, Epstein-Barr virus-transformed B cells, and hematopoietic progenitor cells (14, 24, 32, 54). It is also involved in differentiation of B cells, T cells, macrophages, and neuronal cells (21, 38, 39, 42, 46, 52). Finally, IL-6 stimulates hepatocytes to produce acute-phase reactants (15).

The signal transduction pathways generating these diverse IL-6 responses are not known. IL-6 initiates its biological effects through interaction with specific membrane receptors on target cells (11, 51). Human IL-6 and murine IL-6 as well as the ligand binding subunit of the human IL-6 receptor have been cloned (7, 16, 22, 63, 66). However, the primary sequence of the 80-kDa ligand binding subunit of the IL-6 receptor does not contain any kinase domain or exhibit homology to any other known cytokine receptor and therefore has not provided insights into the signal transduction pathways in IL-6-induced cellular responses. Recently, it has been reported that IL-6 binding to the 80-kDa IL-6 receptor subunit promotes formation of a complex with a 130-kDa non-ligand binding glycoprotein possibly involved in signal transduction (50). To resolve the molecular mechanisms involved in the multiple effector functions of IL-6, we examined the signaling pathways and the programmed expression of early response genes leading to IL-6-stimulated differentiation or proliferation in different cell systems.

Here we report that IL-6 activation of B-cell hybridoma and plasmacytoma proliferation proceeds through a new protein kinase signaling pathway unlike any now known for other cytokines or growth factors. IL-6 stimulation of IL-6-deprived cells resulted in the rapid and transient tyrosine phosphorylation of a cytoplasmic 160-kDa protein (p160). This tyrosine kinase activity was followed by a second protein kinase activity which was inhibited by the potent protein kinase inhibitor H7. This H7-sensitive kinase activity did not correspond to any of the well-characterized protein kinase-second messengers (including protein kinase C [PKC], cyclic AMP [cAMP]-dependent kinase [PKA], cGMP-dependent kinase, or Ca^{2+}/calmodulin-dependent kinase) or to Raf-1, microtubule-associated protein (MAP) kinase, or casein kinase II (2, 33, 48). The results of kinetic and protein kinase inhibitor studies indicated that both the tyrosine kinase and H7-sensitive protein kinase activities are necessary for the highly selective transcriptional activation of two primary response genes, TIS11 (31, 58) and the AP-1 transcription factor, *junB* (44). Nine other primary response genes tested were not induced by IL-6 treatment of MH60.BSF-2 hybridoma B cells. IL-6-induced TIS11 and *junB* transcription was not blocked by cycloheximide, suggesting that posttranslational processes, likely involving direct phosphorylation through the IL-6-induced protein kinase cascade, turn on these primary response genes.

MATERIALS AND METHODS

Reagents. The sources for some of the materials used were as follows: 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), *N*6,2′-*O*-dibutyryl cAMP (dBcAMP), 8-bromo-cGMP (8-Br-cGMP), cholera toxin, and calcium ionophore A23187 were from Sigma Chemical Co. (St. Louis, Mo.); protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-metylpiperazine (H7) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) were from Seikagaku America (St. Petersburg, Fla.); sphingosine was from Sigma; genistein was from Biomol Research Laboratories (Plymouth Meeting, Pa.); tyrphostin (RG50863) was kindly supplied by Rorer Central Research (Philadelphia, Pa.).

Cell culture. MH60.BSF-2 B-cell hybridoma cells (36) were grown in RPMI 1640 medium with 10% fetal calf serum in the presence of 1 ng of IL-6 per ml. A subclone of MH60.BSF-2 growing without IL-6 (and unresponsive to

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IL-6) was obtained from E. C. Breen. Plasmacytoma T1165 cells were from R. Nordan (National Institutes of Health). MH60.BSF-2 cells are strictly dependent on IL-6 for growth. No other factors tested, including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, beta interferon, gamma interferon, or G-CSF, supported the survival and growth of this cell line (36). MH60.BSF-2 cells retained >95% viability after deprivation of IL-6 for 15 to 17 h. Complete loss of cell viability was observed after 48 h without IL-6.

Cell labeling and immunoprecipitation of phosphotyrosine-containing proteins. MH60.BSF-2 cells (10^7/ml) which had been deprived of IL-6 for 12 h or an IL-6-independent subclone of MH60.BSF-2 cells were labeled for 2 h in phosphate-free modified minimal essential medium (supplemented with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], nonessential amino acids, 10% heat-inactivated fetal calf serum dialyzed against 150 mM NaCl) with 1 mCi of carrier-free $^{32}PO_4$ (ICN, Irvine, Calif.). ^{32}P-labeled cells (10^7) were stimulated with IL-6 for various periods, washed with cold phosphate-buffered saline once, and lysed in 1 ml of Nonidet P-40 lysis buffer. Cytoplasmic supernatants were incubated with the antiphosphotyrosine monoclonal antibody 1G2 coupled to Sepharose (23). Phosphotyrosine-containing proteins were eluted with 1 mM phenylphosphate. The eluate from 5×10^6 cell equivalents (about 25 μl) was mixed with an equal volume of 2× sodium dodecyl sulfate (SDS)-sample buffer–5% 2-mercaptoethanol, heated for 5 min, and analyzed on SDS 7.5% polyacrylamide gels.

Immunoblotting of phosphotyrosine-containing proteins. IL-6-deprived MH60.BSF-2 cells (10^7/ml) were stimulated with 300 ng of IL-6 per ml for various times at 37°C and washed with ice-cold Tris-buffered saline. Protein sample buffer (1 ml) (5 mM sodium phosphate, [pH 6.8], 2% SDS, 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue) at 100°C was added to each sample. The sample was pipetted and boiled for 5 min, sheared ten times through a 25-gauge needle, and stored at −70°C prior to use. Immunoblotting was performed essentially by the method of Kamps and Sefton (26). Electrophoretically separated proteins were transferred to Immobilon P (Millipore Corp., Bedford, Mass.). Affinity-purified rabbit polyclonal antibodies to phosphotyrosine were generously provided by J. Ledbetter (Oncogene Corp., Seattle, Wash.) and were used in immunoblotting at 0.5 μg/ml. Rabbit antibodies were detected by incubating immunoblotted filters with ^{125}I-labeled protein A at 1 μCi/ml.

RNA isolation and analysis. Total cytoplasmic RNA was prepared by the Nonidet P-40–proteinase K method (35). RNA was either separated by electrophoresis in 1% agarose-MOPS (morpholinepropanesulfonic acid)-formaldehyde gel and transferred to nitrocellulose membranes (Millipore, type HA) or directly applied to nitrocellulose membranes with the Minifold II slot-blot apparatus (Schleicher & Schuell). Hybridizations were performed at 65°C (60). Membranes were washed at 55°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. Probes were labeled by the random oligonucleotide primer labeling method. Primary response gene probes used were TIS11 (1.6-kb *Eco*RI fragment of pTIS11 from H. Herschman), *junB* (1.8-kb *Eco*RI fragment of p465.20 from D. Nathans), c-*myc* (1.6-kb exon 3-specific *Sst*I-*Hin*dIII fragment from K. Calame), c-*fos* (1.0-kb *Pst*I fragment of pfos-1 [v-*fos*] from American Type Culture Collection), c-*jun* (0.95-kb *Eco*RI-*Bam*HI fragment from P. Vogt), TIS8/*Egr-1*/NGFI-A/Krox24 (1.3-kb *Eco*RI fragment of pTIS8 from H. Herschman), *Egr-2*/Krox20 (2.3-kb *Eco*RI fragment of pAC16 from P. Charnay), *fra-1* (1.5-kb *Eco*RI fragment of PSP65-*fra-1* from T. Curran and D. Cohen), and *fosB* (2.1-kb *Eco*RI fragment of AC113-1 from R. Bravo). Plasmids for pTIS1, pTIS7, and pTIS10 primary response gene probes (31) were obtained from H. Herschman. CHO-B (0.6-kb *Eco*RI-*Bam*HI fragment [18] from J. Darnell, Jr.) was used as an internal standard for normalization of RNA samples. All labeled DNA probes were shown to hybridize with RNA isolated from serum-stimulated NIH 3T3 cells.

Nuclear transcription assays. Preparation of nuclei and transcription reactions were performed as previously described (60). Labeled RNA was purified and hybridized for 48 h at 65°C with denatured DNAs (3 μg per slot) on nitrocellulose membranes. Before washing, hybridized membranes were incubated with 2.5 μg of RNase A per ml and 5 U of RNase T_1 per ml in 2× SSC for 30 min at 37°C.

RESULTS

IL-6 rapidly induces tyrosine phosphorylation of 160-kDa protein. We first examined the effects of a panel of protein kinase activators of known signal transduction pathways (in the presence and absence of IL-6) on the growth of IL-6-deprived MH60.BSF-2 cells. No activator of PKC, PKA, or cyclic nucleotide- or Ca^{2+}/calmodulin-dependent protein kinase tested promoted the growth of MH60.BSF-2 cells in the absence of IL-6 (data not shown). Depletion of PKC by long-term (i.e., 48-h) TPA also had no effect on IL-6-induced hybridoma cell growth. No tested combination of activators maintained cell survival or enhanced IL-6-induced cell growth, making it unlikely that IL-6 acts via multiple kinase pathways by using known second messenger systems (data not shown). From these combined results, we concluded that none of the major signaling pathways utilizing well-characterized second messenger systems (PKC, PKA, cGMP-dependent kinase, Ca^{2+}/calmodulin-dependent kinases) are required for IL-6-activated signal transduction in the growth of MH60.BSF-2 hybridoma cells.

In addition to serine/threonine kinases such as PKC, PKA, and Ca^{2+}/calmodulin-dependent kinases, membrane-associated tyrosine kinases (both receptor and nonreceptor types) play important roles in the signal transduction pathways of growth and differentiation factors. To test the possible involvement of tyrosine kinases in IL-6-induced proliferation, we used antiphosphotyrosine-specific monoclonal antibody to screen for tyrosine-phosphorylated proteins following IL-6 addition to IL-6-deprived MH60.BSF-2 cells. In these experiments, IL-6-deprived MH60.BSF-2 cells or an IL-6-independent line of MH60.BSF-2 cells were labeled with $^{32}PO_4$ in phosphate-free medium for 2 h and then stimulated with IL-6 for various times. Phosphotyrosine-containing proteins were immunoprecipitated from labeled cell lysates with monoclonal antiphosphotyrosine antibody 1G2 (23) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Fig. 1A). IL-6 rapidly induced the transient appearance of one new cellular phosphoprotein of 160 kDa (p160) in IL-6-dependent MH60.BSF-2 cells (lanes 2 and 3) but not in the IL-6-independent variant of MH60.BSF-2 cells (lanes 6 and 7). This tyrosine-phosphorylated p160 protein appeared within 2 min, reached a maximal level at 5 min, and thereafter rapidly decreased (Fig. 1B). This rapid and transient stimulation of phosphorylation is a typical response to growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (13, 62). IL-6

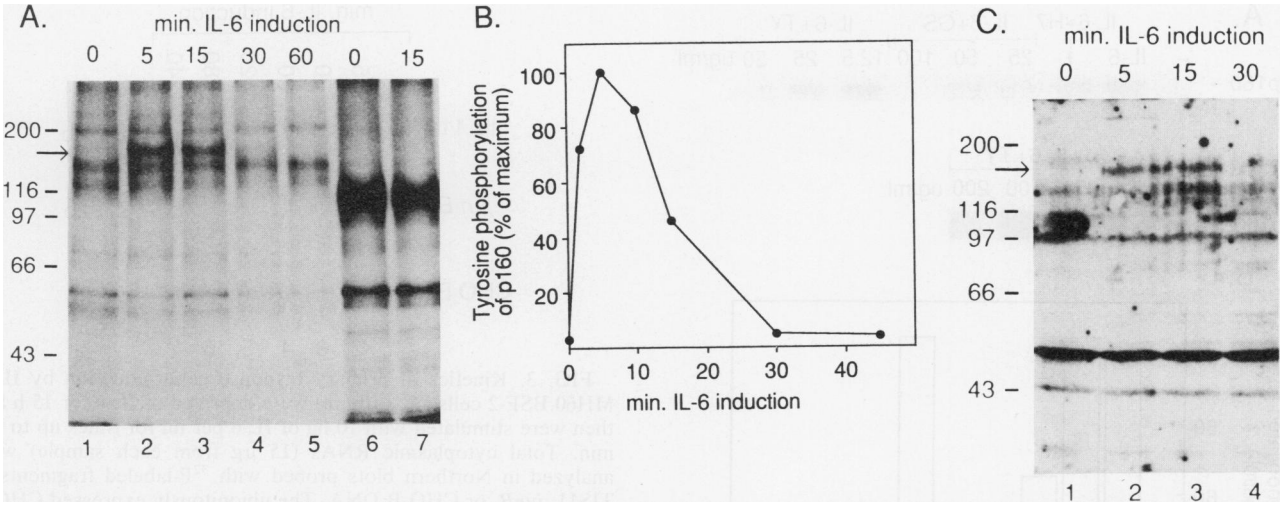

FIG. 1. Tyrosine phosphorylation of cellular proteins in response to IL-6. (A) Immunoprecipitation of pulse-labeled phosphotyrosine proteins. IL-6-deprived MH60.BSF-2 cells (lanes 1 to 5) and an IL-6-independent subclone of MH60.BSF-2 cells (lanes 6 and 7) were labeled for 2 h with 1 mCi of $^{32}PO_4$ per ml in phosphate-free medium containing 10% dialyzed fetal calf serum and then stimulated with 300 ng of IL-6 per ml for the periods indicated. Phosphotyrosine-containing proteins were immunoprecipitated from cytoplasmic supernatants with monoclonal antiphosphotyrosine antibody 1G2 (23) coupled to Sepharose 4B and analyzed by SDS-7.5% PAGE under reducing conditions. Numbers on left show size in kilodaltons. (B) Kinetics of IL-6-induced tyrosine-phosphorylated p160 protein. Results were compiled from four separate kinetic labeling experiments. Results are plotted as percent maximum labeled p160 obtained at 5 min after IL-6 stimulation. (C) Immunoblots of phosphotyrosine-containing proteins. Whole-cell extracts from IL-6-deprived MH60.BSF-2 cells, unstimulated or IL-6 stimulated for the times indicated, were analyzed by SDS-7.5% PAGE under reducing conditions, transferred to an Immobilon P membrane, and probed with antiphosphotyrosine polyclonal antiserum (26). Incubation with either phosphotyrosine or phenylphosphate inhibited the subsequent immunoprecipitation of p160 by 1G2 antibody (23) or the detection of p160 in immunoblots with antiphosphotyrosine antiserum (26) (data not shown).

increased the amount of tyrosine-phosphorylated p160 in a dose-dependent manner with a maximum response at 100 ng/ml (5 nM) when assayed after 5 min of stimulation (data not shown). No change in tyrosine-phosphorylated proteins was detected in the IL-6-independent subclone of MH60.BSF-2 cells (lanes 6 and 7), which does not make or respond to IL-6. The growth of this IL-6-independent cell line may be signaled by a different pathway or through an activated signaling step downstream of tyrosine kinase phosphorylation.

We also did protein gel immunoblotting using polyclonal antiphosphotyrosine antibody to directly identify any changes in tyrosine-phosphorylated proteins following IL-6 induction (26). In four separate immunoblotting experiments, the 160-kDa protein was the only band which consistently and reproducibly showed increased tyrosine phosphorylation following IL-6 stimulation (Fig. 1C). The p160 band detected by antiphosphotyrosine antibody immunoblotting exhibited the same kinetics observed in the monoclonal antibody immunoprecipitation experiments (compared Fig. 1A and C). Apparent changes in bands at approximately 140 and 100 kDa in Fig. 1C were not seen in repeated immunoblots with this polyclonal antiphosphotyrosine antibody. These findings confirm that the observed IL-6 induction of tyrosine-phosphorylated p160 detected by monoclonal antibody immunoprecipitation reflects tyrosine phosphorylation of the p160 protein. These results show that p160 is the primary substrate phosphorylated by the IL-6-stimulated tyrosine kinase. It is possible that the use of other antiphosphotyrosine antibodies with different specificities may reveal additional IL-6-inducible tyrosine-phosphorylated proteins. No phosphorylation of p160 was observed after a 60-min incubation at 4°C (data not shown). This result is different from the tyrosine phosphorylation response to PDGF, EGF, or colony-stimulating factor-1 (13, 47, 64), suggesting the existence of processes intervening between the IL-6 receptor complex and the IL-6-activated tyrosine kinase.

Tyrosine phosphorylation of p160 is inhibited by the specific tyrosine kinase inhibitor genistein or tyrphostin but not by the general kinase inhibitor H7. We next examined the effects of two tyrosine kinase inhibitors with different modes of action, genistein (1) and tyrphostin (62), on the IL-6-induced tyrosine phosphorylation of p160. Akiyama et al. (1) reported that genistein specifically inhibited tyrosine kinase activities of EGF receptor, pp60$^{v\text{-}src}$, and pp110$^{gag\text{-}fes}$ by competing with ATP (in vitro 50% inhibitory dose [ID_{50}], 6 to 8 μg/ml). The genistein inhibition of tyrosine kinase activity was highly specific, since the in vitro ID_{50} for serine/threonine kinases including PKC, PKA, phosphorylase kinase, 5′-nucleotidase, and phosphodiesterase were higher than 100 μg/ml. Tyrphostin is the general name for derivatives of cyanobenzylidene with specific inhibitory effects on EGF receptor tyrosine kinase and with little effect on the insulin receptor kinase (62). Tyrphostins compete with phosphorylation substrates at the substrate binding site of the EGF receptor tyrosine kinase. One tyrphostin reportedly inhibited PDGF-induced cell proliferation (34), suggesting that certain of these compounds inhibit other tyrosine kinases. Tyrphostins only minimally affect the activities of PKC and PKA.

Genistein or tyrphostin (RG50863) was added to ^{32}P-labeled MH60.BSF-2 cells 30 min prior to IL-6 stimulation for 5 min. Both compounds effectively inhibited the IL-6-induced tyrosine phosphorylation of p160 (Fig. 2A and B). Genistein at 50 and 100 μg/ml inhibited p160 phosphorylation by 60 and 95%, respectively. Tyrphostin at 50, 100, and 200 μg/ml inhibited p160 phosphorylation by 60, 75, and 90%,

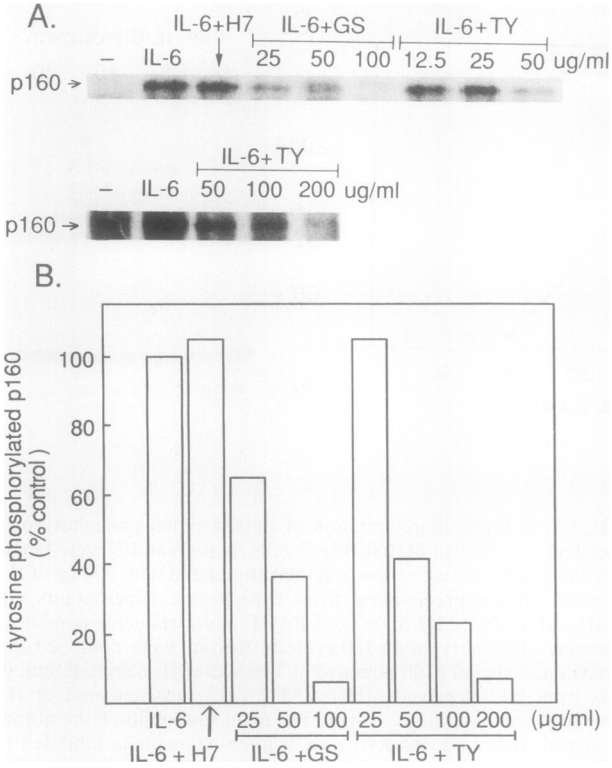

FIG. 2. Effect of protein kinase inhibitors on p160 phosphorylation. (A) Electrophoretic analyses of inhibitor effects. $^{32}P_i$-labeled MH60.BSF-2 cells (10^7/ml) were treated with genistein (GS) (25 to 100 μg/ml), tyrphostin (TY) (12.5 to 200 μg/ml), or H7 (100 μM) for 30 min and then stimulated with IL-6 (300 ng/ml) for 5 min. Control cells were stimulated with IL-6 alone. Tyrosine-phosphorylated proteins were analyzed as described in the legend to Fig. 1. Only the p160 region of the SDS-polyacrylamide gels is shown. Two representative experiments from four separate determinations are shown. Levels of p160 were determined by densitometry. (B) Quantitative effects of inhibitors on p160 phosphorylation. Results are expressed as percentages of the responses to IL-6 without inhibitor averaged from four experiments. Solvent alone (solvent used, 0.1% DMSO plus 0.5% methanol for genistein, 0.1% DMSO for tyrphostin) did not affect the level of p160 induced by IL-6 (data not shown).

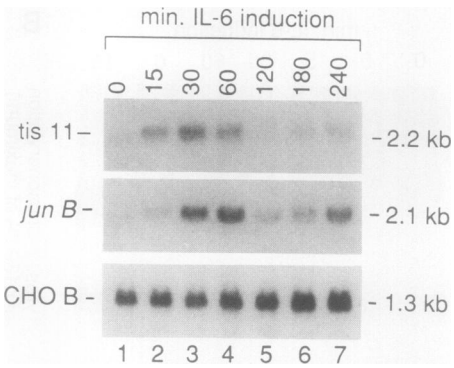

FIG. 3. Kinetics of primary response gene induction by IL-6. MH60.BSF-2 cells (3×10^5/ml) were deprived of IL-6 for 15 h and then were stimulated with 10 ng of IL-6 per ml for times up to 240 min. Total cytoplasmic RNAs (15 μg from each sample) were analyzed in Northern blots probed with ^{32}P-labeled fragments of TIS11, *junB*, or CHO-B DNA. The ubiquitously expressed CHO-B gene was used as an internal standard for normalization of samples. Nine other primary response genes tested were not detectably induced by IL-6 including c-*jun*, c-*fos*, TIS1 (*nur-77* or NGFIB), TIS7 (PC4), TIS8 (*Egr-1*, NGFIA, *zif-286*, Krox24), *fra-1*, *fosB*, TIS10, and Krox20 (data not shown). All primary response gene probes were shown to hybridize with RNA from serum-stimulated NIH 3T3 cells.

respectively. Tyrphostin inhibited IL-6-stimulated tyrosine kinase activity as effectively as genistein, confirming that this inhibitor affects tyrosine kinases other than the EGF receptor.

We next asked whether the potent, nonspecific protein kinase inhibitor H7 blocked IL-6-activated tyrosine phosphorylation of p160. H7 is known to inhibit PKC, PKA, and cGMP-dependent protein kinases (20). The results in Fig. 2A and B show that H7 at 100 μM did not inhibit the tyrosine phosphorylation of p160. This indicates that the rapid tyrosine phosphorylation of p160 following IL-6 binding to the IL-6 receptor does not require prior activation of any H7-sensitive protein kinase(s).

IL-6 selectively induces the expression of two primary response genes, *junB* and TIS11. We next examined IL-6-induced hybridoma cells for the expression of primary response genes. Such genes are rapidly expressed in response to a variety of inducing agents and are believed to play important roles in controlling cellular growth and differentiation. We tested the IL-6-induced responses of 11 such genes including c-*fos* (12, 55), c-*jun* (3, 5), *junB* (44), *Egr-2* (also called Krox20) (6), TIS11 (31), TIS1 (also called *nur-77* and NGFIB) (19, 31, 37), TIS7 (or PC4) (31, 53, 59), *Egr-1* (also called TIS8, NGFIA, *zif-286*, and Krox24) (9, 30, 31, 37, 49), TIS10 (31), *fra-1* (10), and *fosB* (65).

Total cellular RNA was obtained from MH60.BSF-2 cells which had been deprived of IL-6 for 15 h and then stimulated with IL-6 for up to 240 min. Northern (RNA) blot analyses showed that both TIS11 and *junB* mRNAs were elevated 10- to 15-fold between 30 and 60 min after IL-6 addition (Fig. 3, lanes 2 to 4). The elevated levels of TIS11 and *junB* mRNAs then declined gradually (lanes 5 to 7) up to 240 min. Slightly elevated levels of mRNAs were present even after 10 h of stimulation and in normally growing MH60.BSF-2 cells in the presence of IL-6 (data not shown). The activation of TIS11 and *junB* genes by IL-6 was also observed with similar kinetics in an IL-6-dependent plasmacytoma cell line, T1165 (40a).

The induction of TIS11 and *junB* mRNAs in IL-6-treated MH60.BSF-2 cells was extremely selective. The results in Fig. 4 reaffirm that IL-6 increased *junB* (lane 3) and TIS11 (lane 6) mRNAs but had no effect on either c-*fos* (lane 9) or *Egr-1* (lane 12) mRNA. IL-6 also had no effect on seven other primary response genes tested (c-*jun*, TIS1, TIS7, TIS10, *Egr-2*, *fra-1*, *fosB*) (unpublished data). TPA treatment of MH60.BSF-2 cells induced *junB*, TIS11, c-*fos*, and *Egr-1* mRNAs (lanes 2, 5, 8, and 11, respectively) as well as *Egr-2* mRNA (data not shown). The different spectrum of primary response genes induced in MH60.BSF-2 cells by TPA versus IL-6 strongly suggests that IL-6 signaling is not mediated by PKC. Multiple primary response genes are typically activated by stimuli which induce cell proliferation. In this regard, the selective induction of only 2 of 11 primary response genes by IL-6 is unusual.

Posttranslational mechanisms activate *junB* and TIS11 transcription. We did in vitro transcription assays to examine the mechanisms involved in the IL-6-induced increases in TIS11 and *junB* mRNA. In these studies, we compared the effect of cycloheximide on IL-6-induced TIS11 and *junB* gene tran-

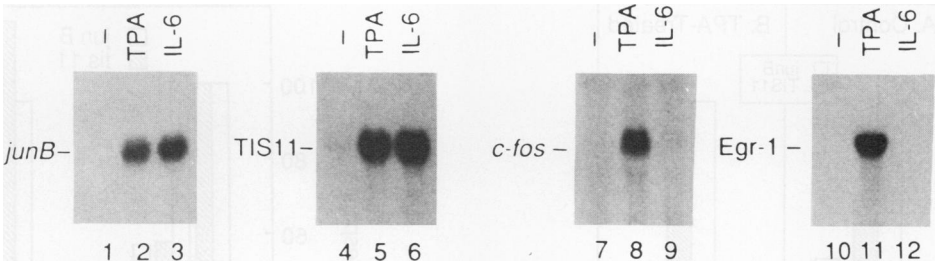

FIG. 4. Primary response genes induced in IL-6- or TPA-treated MH60.BSF-2 cells. Total cytoplasmic RNA from MH60.BSF-2 cells, either unstimulated or stimulated with IL-6 (10 ng/ml) for 30 min, was analyzed in Northern blots probed with labeled *junB* (lanes 1 to 3), TIS11 (lanes 4 to 6), c-*fos* (lanes 7 to 9), and *Egr-1* (lanes 10 to 12) DNAs.

scription in nuclear run-on transcription assays (Fig. 5). IL-6-deprived MH60.BSF-2 cells were stimulated with IL-6 for either 30 min or 3 h (lanes 2 and 4) or with IL-6 plus cycloheximide for 30 min after a 30-min pretreatment with cycloheximide (lane 3). IL-6 stimulation for 30 min induced TIS11 and *junB* transcription 12- to 14-fold (lane 2) over that in uninduced control cells (lane 1). These values equal the IL-6-induced increases in TIS11 and *junB* mRNAs (Fig. 3), indicating that transcriptional activation is the major mechanism responsible for the IL-6-induced increases in these two primary response genes. Interestingly, cycloheximide did not block IL-6-induced transcription of TIS11 or *junB* (lane 3). Both genes were induced 13- to 15-fold by a 30-min induction with IL-6 in the presence of cycloheximide. This finding suggests that posttranslational mechanisms alone can account for the IL-6-induced increases in transcription of the TIS11 and *junB* genes. Treatment of cells with cycloheximide alone for 30 min induced only modest increases in *junB* and TIS11 transcription (1.8- and 1.3-fold, respectively) (data not shown). After 3 h of IL-6 stimulation, TIS11 and *junB* gene transcription decreased to a level only two- to fourfold (lane 4) higher than that in unstimulated control cells. This low level of transcription is comparable to the level of TIS11 and *junB* mRNA seen 3 to 4 h after induction (Fig. 3, lanes 6 and 7). This suggests that transcriptional down-regulation is the primary mechanism causing the decline in TIS11 and *junB* mRNA.

We next tested the possible involvement of known second messengers in TIS11 and *junB* induction. TIS11 and *junB* mRNA levels were assayed after 45 min of treatment with TPA, dBcAMP, cholera toxin, 8-Br-cGMP, A23187, or IL-6 (Fig. 6). TIS11 and *junB* mRNAs were stimulated between 12- and 18-fold by IL-6. Neither gene was activated by dBcAMP, cholera toxin, 8-Br-cGMP, or A23187 in this cell line (Fig. 6). The stimulation with these agents was extended to 6 h without detectable TIS11 or *junB* mRNA induction. TPA induced a 3- to 7-fold increase in mRNA from both genes (Fig. 6). In a separate experiment, MH60.BSF-2 cells were treated with TPA (in 0.1% dimethyl sulfoxide [DMSO]) for 40 h to deplete PKC. Control cells were treated with 0.1% DMSO alone. TPA-treated and control cells were tested for IL-6- or TPA-induced TIS11 and *junB* mRNA induction (Fig. 7). Successful depletion of PKC activity by

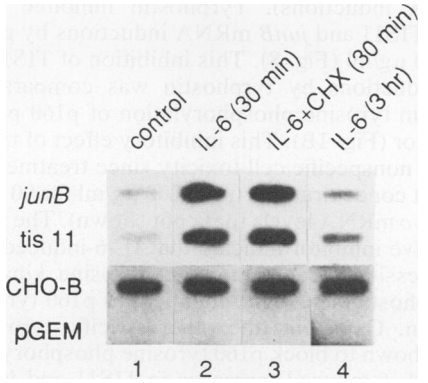

FIG. 5. IL-6 induces TIS11 and *junB* gene transcription. Nuclear run-on transcription assays (60) were done on nuclei prepared from IL-6-deprived MH60.BSF-2 cells without stimulation (lane 1, control), stimulated with 10 ng of IL-6 per ml for 30 min (lane 2), pretreated with 10 μg of cycloheximide (CHX) per ml for 30 min followed by IL-6 stimulation for 30 min (lane 3), or stimulated with IL-6 for 3 h (lane 4). Isolated labeled RNA was hybridized with 3 μg of linearized DNA containing TIS11, *junB*, or CHO-B DNA or pGEM1 plasmid DNA as a control.

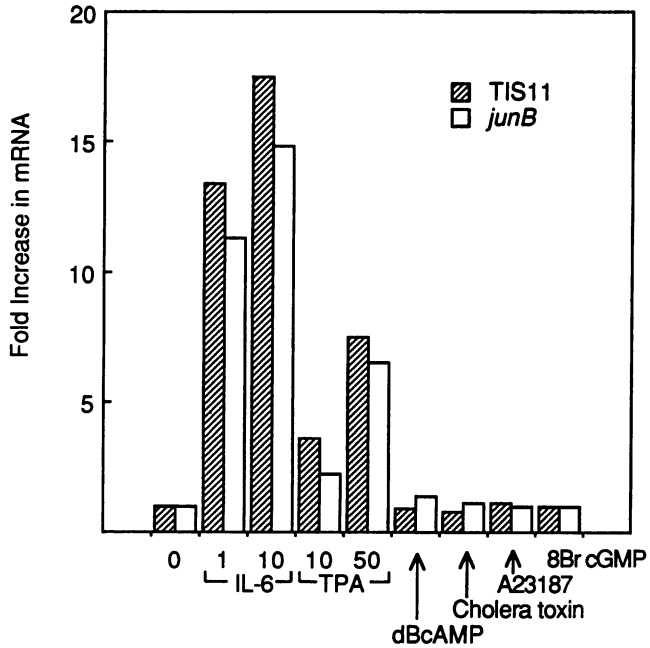

FIG. 6. Analysis of TIS11 and *junB* induction by IL-6 and known protein kinase activators. IL-6-deprived cells (3×10^5/ml) were stimulated with IL-6 (1 and 10 ng/ml), TPA (10 and 50 ng/ml), dBcAMP (1 mM), cholera toxin (1 μg/ml), calcium ionophore A23187 (1 μM), or 8-Br-cGMP (1 mM) for 45 min. The levels of TIS11 and *junB* mRNAs were measured by slot-blot hybridization.

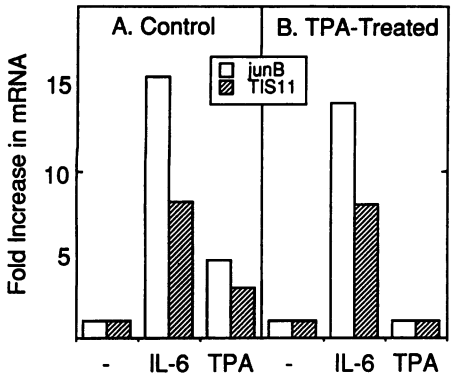

FIG. 7. Effect of PKC depletion on TPA- or IL-6-induced TIS11 and *junB* expression. Control MH60.BSF-2 cells incubated with medium containing 0.1% DMSO alone (A) or stimulated with TPA (1 μg/ml in medium with 0.1% DMSO) for 40 h (B) were deprived of IL-6 for the final 16 h and then induced with either IL-6 (10 ng/ml) or TPA (50 ng/ml) for 45 min. Total cytoplasmic RNA samples were isolated and hybridized in slot blots with labeled TIS11 or *junB* DNA. Results are presented as the fold increase in mRNA over levels in unstimulated (−) cells. PKC activity was measured in DEAE-Sephacel-fractionated extracts of control cells or TPA-treated cells by using a PKC assay kit from Amersham. Cell extracts from control cells exhibited a PKC activity of 52 pmol/min/25 μl, whereas extracts from TPA-treated cells showed >0.13 pmol/min/25 μl, confirming that the 40-h TPA treatment had effectively depleted PKC activity.

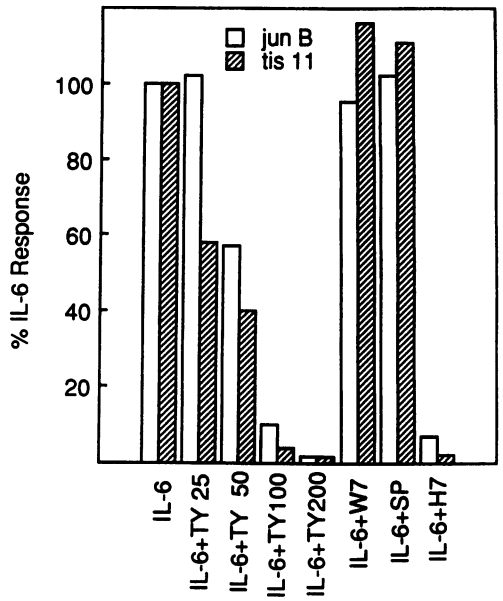

FIG. 8. Effect of protein kinase inhibitors on IL-6-induced TIS11 and *junB* gene expression. IL-6-deprived MH60.BSF-2 cells (10^7 cells per ml) were treated with tyrphostin (TY) at 25 to 200 μg/ml for 30 min prior to IL-6 stimulation. Untreated control cells and tyrphostin-treated cells were stimulated with IL-6 at 300 ng/ml for 45 min. In studies with other inhibitors, IL-6-deprived MH60.BSF-2 cells (3×10^5 cells per ml) were pretreated with H7 (20 μM), sphingosine (10 μM), or W7 (40 μM) for 30 min, followed by stimulation with 1 ng of IL-6 per ml for 45 min. The effects of different inhibitors on TIS11 or *junB* mRNA induction were analyzed in slot blots and quantitated by densitometry. The levels of TIS11 or *junB* mRNA are expressed as percentages of the IL-6-induced levels without inhibitor. IL-6 at 1 ng/ml increased TIS11 mRNA by 14-fold and *junB* mRNA by 10-fold over basal levels in IL-6-deprived cells. Sphingosine at 10 μM completely inhibited TPA-induced (50 ng/ml) TIS11 and *junB* gene expression (data not shown).

40 h of TPA treatment was confirmed by direct PKC assays (Amersham). Restimulation with TPA failed to increase TIS11 and *junB* mRNA in cells depleted of PKC by long-term TPA treatment (Fig. 7B). In contrast, IL-6 stimulation of PKC-depleted cells induced increases in TIS11 and *junB* mRNAs identical to those in IL-6-induced control cells (Fig. 7A and B). These results collectively suggest that IL-6-activated expression of TIS11 and *junB* genes occurs via a signaling pathway distinct from PKC, PKA, cGMP-dependent kinase, or Ca^{2+}/calmodulin-dependent kinases. Accordingly, the induction of TIS11 and *junB* gene expression shows the same pattern of responsiveness as IL-6-stimulated cell proliferation.

Tyrosine kinase and H7-sensitive kinase activities are required for IL-6-induced TIS11 and *junB* gene expression. To further resolve the IL-6 signaling pathway(s) leading to immediate response gene activation, we tested various protein kinase inhibitors on IL-6-induced TIS11 and *junB* gene expression. TIS11 and *junB* genes are suitable target genes for this approach because their induction by IL-6 does not require new protein synthesis. The rapid response of these genes to IL-6 also made it possible to limit exposure time to various protein kinase inhibitors, thereby minimizing nonspecific or toxic effects possibly causing general inhibition of transcription. Levels of c-*myc* mRNA were monitored since the 2.0- and 1.85-kb c-*myc* mRNA species (transcribed from translocated c-*myc*) were high in IL-6-deprived MH60.BSF-2 cells and reflective of general transcription rates because of the short half-life of these mRNAs ($T_{1/2}$ = ~30 min measured in the absence of IL-6) (40a). Protein kinase inhibitors were added 30 min before treatment with IL-6. MH60.BSF-2 cells were harvested after IL-6 stimulation for 45 min, and total cellular RNA was analyzed for TIS11 and *junB* mRNA in slot blots.

The effect of the tyrosine kinase inhibitor tyrphostin on IL-6-induced TIS11 and *junB* mRNA expression was examined under the same conditions used to inhibit p160 tyrosine phosphorylation (except that a 45-min stimulation was used for mRNA inductions). Tyrphostin inhibited both IL-6-activated TIS11 and *junB* mRNA inductions by greater than 90% at 100 μg/ml (Fig. 8). This inhibition of TIS11 and *junB* mRNA inductions by tyrphostin was comparable to the reduction in tyrosine phosphorylation of p160 produced by this inhibitor (Fig. 1B). This inhibitory effect of tyrphostin is not due to nonspecific cell toxicity since treatment with this inhibitor at concentrations up to 200 μg/ml for 90 min did not affect c-*myc* mRNA levels (data not shown). The results with this selective inhibitor indicate that IL-6-induced TIS11 and *junB* expression is dependent on tyrosine kinase activity with tyrophostin sensitivity identical to p160 tyrosine phosphorylation. Genistein (the other specific tyrosine kinase inhibitor shown to block p160 tyrosine phosphorylation) also inhibited IL-6-induced increases in TIS11 and *junB* mRNA (data not shown). However, a 90-min treatment with genistein at >100 μg/ml also reduced c-*myc* mRNA levels, suggesting that cell toxicity contributes to the results seen with this inhibitor.

We also examined the effect of other protein kinase inhibitors on IL-6-induced TIS11 and *junB* gene expression (Fig. 8). Sphingosine, an inhibitor of PKC (17), completely inhibited the induction of TIS11 and *junB* expression by TPA

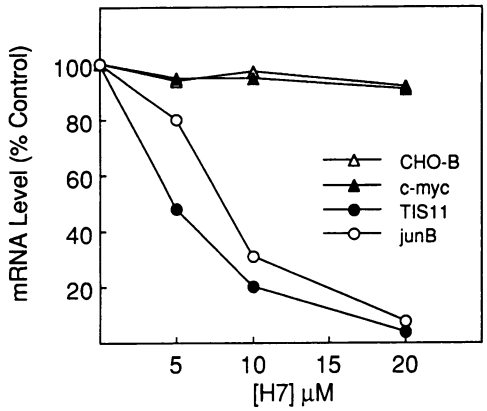

FIG. 9. Dose-response curve for H7 inhibition of TIS11 and *junB* induction. IL-6-deprived MH60.BSF-2 cells were incubated with different concentrations of H7 for 30 min and then stimulated with IL-6 for 45 min as described in the legend to Fig. 8. Cells used for c-*myc* mRNA analyses were treated with H7 alone. Levels of TIS11, *junB*, CHO-B, and c-*myc* mRNAs were determined in slot blots.

(data not shown) but had no effect on IL-6-stimulated TIS11 and *junB* gene expression. As expected from the fact that the calcium ionophore A23187 failed to induce TIS11 gene expression, the calmodulin antagonist W7 (20) had no effect on IL-6-induced TIS11 or *junB* mRNA expression (Fig. 8). Unexpectedly, the induction of TIS11 and *junB* mRNA was completely blocked by the kinase inhibitor H7 at 20 μM (Fig. 9). The levels of c-*myc* and CHO-B mRNAs in this experiment were not detectably altered (even at the highest H7 concentration), indicating that this effect on TIS11 and *junB* induction did not result from general H7 toxicity. This latter finding reveals that the IL-6 signaling pathway leading to activation of the TIS11 and *junB* genes is dependent on the activity of an H7-sensitive kinase.

The combined results of activator and inhibitor studies described here suggest that this H7-sensitive kinase is distinct from the major signal transduction pathways utilizing known mediators such as diacylglycerol, Ca^{2+}, cAMP, or cGMP. Thus, these two different primary response genes appear to be coordinately regulated through a common IL-6-activated signal transduction pathway requiring the activities of a tyrosine kinase and a kinase (or kinases) sensitive to the protein kinase inhibitor H7.

DISCUSSION

IL-6-induced hybridoma B-cell growth is activated through a novel signal transduction pathway. We determined that IL-6 activates an intracellular signal transduction pathway which apparently does not utilize any known second messengers (e.g., PKC, PKA, cGMP-dependent kinase, Ca^{2+}/calmodulin kinases), either alone or in combination. Within minutes of IL-6 binding at 37°C, we detected the tyrosine phosphorylation of a cellular protein with the apparent molecular mass of 160 kDa (p160). Unlike EGF and PDGF, which rapidly induce significant tyrosine phosphorylation of several proteins at 4°C, IL-6 does not stimulate p160 tyrosine phosphorylation at this temperature. This difference probably reflects the nature and physical relationship of these tyrosine kinases with their relevant receptor molecules. Both EGF receptor and PDGF receptor molecules contain tyrosine kinase domains, while the 80-kDa ligand binding subunit of the IL-6 receptor has been shown not to have a tyrosine kinase domain (63). It has not been reported whether the recently identified gp130 cellular protein which complexes with the 80-kDa ligand binding subunit of the IL-6 receptor (50) contains a tyrosine kinase domain. Receptor binding studies using ^{125}I-labeled recombinant IL-6 showed the existence of two classes of IL-6 receptors with two different affinities (63). It is possible that the IL-6 receptor complex contains a yet unidentified component with a tyrosine kinase domain. Resolving the nature of the tyrosine kinase which phosphorylates p160 will be an important step in further deciphering IL-6-induced signal transduction.

Perhaps the most interesting finding of our studies is the linkage of IL-6-induced intracellular signal transduction to the highly selective induction of two primary response genes, TIS11 and *junB*. The TIS11 gene appears to code for a nuclear protein, although of unknown function (58). The *junB* gene is one of the *jun*/AP-1 family of nuclear proto-oncogenes known to function in controlling transcription (44). The proto-oncogene c-*jun* (3, 5) encodes the transcription factor AP-1 (4, 29). The related gene products from *junB*, c-*jun*, and *junD* (another member of the *jun* family) dimerize with c-*fos* and bind with increased affinity to AP-1 sites or closely similar sequences (8, 40, 45). The IL-6-induced transcription of both the TIS11 and *junB* genes is unaffected by cycloheximide and therefore appears to be mediated through the posttranslational activation of existing transcription factors presumably through phosphorylation by the IL-6-induced protein kinase cascade indicated in our studies. The initial activation of c-*jun* transcription in TPA-treated cells also occurs by a posttranslational mechanism (3).

The selective activation of these two primary response genes appears to require the activity of an H7-sensitive protein kinase(s) which evidently intercedes between the rapid tyrosine phosphorylation of p160 and TIS11 and *junB* transcription. This proposed sequence of events is supported by the pattern of inhibition by different protein kinase inhibitors. Tyrphostin efficiently blocked IL-6-induced tyrosine phosphorylation of p160 as well as activation of TIS11 and *junB* transcription. In contrast, the potent protein kinase inhibitor H7 blocked TIS11 and *junB* induction but had no effect on p160 tyrosine phosphorylation. The simplest interpretation combining the kinetic events and these inhibitor results is a single IL-6-induced signal transduction pathway leading to the activation of TIS11 and *junB* transcription.

The model in Fig. 10 outlines our proposed pathway for the early steps in IL-6-induced signal transduction in hybridoma and plasmacytoma cells. IL-6 binding results in the association of the 80-kDa IL-6 receptor with gp130 (50). This event activates a novel protein kinase cascade, initiated by tyrosine phosphorylation of a 160-kDa protein (p160), rapidly following IL-6 receptor binding. This process, done by an unidentified tyrosine kinase, then activates an H7-sensitive kinase(s). This H7-sensitive kinase selectively activates the transcription of two primary response genes, TIS11 and the AP-1 transcription factor *junB*. IL-6-induced transcription of the TIS11 and *junB* genes is activated by posttranslational mechanisms which in the simplest case might entail phosphorylation by the H7-sensitive protein kinase(s). Finally, it is highly likely that certain genes which are secondarily induced in response to IL-6 will be activated through *junB* binding to AP-1 or AP-1-related sequences in their promoters or enhancers.

Our results indicate that the H7-sensitive kinase activity required for IL-6-induced TIS11 and *junB* transcription does not correspond to any well-characterized protein kinase.

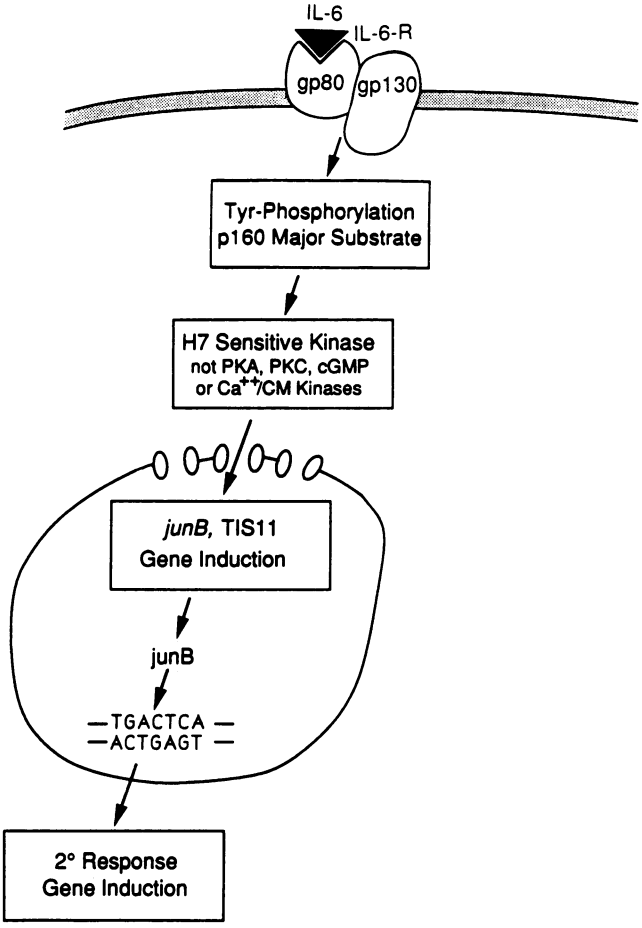

FIG. 10. Model of the early steps in the IL-6 signal transduction pathway. IL-6 binding to 80-kDa ligand binding subunit promotes association with a 130-kDa cellular protein and formation of the IL-6 receptor complex (IL-6-R) (50). This induces tyrosine phosphorylation of p160, which is followed by activation of an H7-sensitive kinase(s) distinct from PKC, PKA, cGMP-dependent kinase, or Ca^{2+}/calmodulin-dependent kinases (Ca^{++}/CM Kinases). The H7-sensitive kinase(s) conveys the IL-6 signal to TIS11 and *junB* gene transcription by posttranslational modification without requiring protein synthesis. It is proposed that the *junB* transcription factor interacts with AP-1 or AP-1-like sites in the genome to activate expression of secondary response genes induced by IL-6.

This conclusion is especially well documented with regard to PKC. Three lines of evidence all show that PKC is not involved in the IL-6-induced intracellular signaling events leading to selective TIS11 and *junB* expression. First, depletion of PKC by long-term TPA treatment eliminated TIS11 and *junB* induction by TPA but had no effect on induction of these primary response genes by IL-6. Second, the specific PKC inhibitor, sphingosine, blocked TIS11 and *junB* induction by TPA but not by IL-6. Third, IL-6 and TPA activated different sets of primary response genes in MH60.BSF-2 hybridoma cells. IL-6 selectively induced only TIS11 and *junB*, whereas TPA induced these two genes as well as c-*fos*, *Egr-1*, and *Egr-2*.

Few protein kinases are known which activate primary response genes without using well-characterized second messenger systems. One possibility is Raf-1 serine/threonine kinase, encoded by the *raf-1* proto-oncogene (43). Expression of the *raf-1* proto-oncogene has been shown to activate the PEA1 transcription factor (which recognizes the AP-1 binding site) (61) and c-*fos* (25). Other protein kinases which are potential candidates for the H7-sensitive activity are MAP kinase (2) and casein kinase II (33, 48). However, in vitro protein kinase assays revealed that IL-6 induction did not activate Raf-1 or either of the other two protein kinases (40a). Accordingly, the H7-sensitive kinase implicated to play a central role in IL-6 activation of TIS11 and *junB* gene transcription remains to be identified.

These studies describe one of the first cytokine activation systems linking intracellular signaling events to the activation and expression of primary response genes. It will be interesting to compare these early signaling steps in IL-6-induced hybridoma and plasmacytoma cell growth with those in IL-6-induced cell growth or differentiation in other cell lineages. We think it is likely that the early activation events defined here also will occur in the diverse IL-6-stimulated responses in different cell lineages. In this case, we would predict that the specific developmental outcomes of IL-6 induction in other cell lineages will be determined by the selective activation of tissue-specific secondary response genes.

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ADDENDUM IN PROOF

The recently cloned gp130 subunit of the complete IL-6 receptor (M. Hibi et al., Cell **63**:1149–1157, 1990) does not contain a tyrosine kinase domain.

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