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Multiple cDNAs Encoding the *esk* Kinase Predict Transmembrane and Intracellular Enzyme Isoforms

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A novel protein kinase, the Esk kinase, has been isolated from an embryonal carcinoma (EC) cell line by using an expression cloning strategy. Sequence analysis of two independent cDNA clones (2.97 and 2.85 kb) suggested the presence of two Esk isoforms in EC cells. The *esk-1* cDNA sequence predicted an 857-amino-acid protein kinase with a putative membrane-spanning domain, while the *esk-2* cDNA predicted an 831-amino-acid kinase which lacked this domain. In adult mouse cells, *esk* mRNA levels were highest in tissues possessing a high proliferation rate or a sizeable stem cell compartment, suggesting that the Esk kinase may play some role in the control of cell proliferation or differentiation. As anticipated from the screening procedure, bacterial expression of the Esk kinase reacted with antiphosphotyrosine antibodies on immunoblots. Furthermore, in in vitro kinase assays, the Esk kinase was shown to phosphorylate both itself and the exogenous substrate myelin basic protein on serine, threonine, and tyrosine residues, confirming that the Esk kinase is a novel member of the serine/threonine/tyrosine family of protein kinases.

The combinatorial actions of serine, threonine, and tyrosine kinases are involved in the regulation of information transfer within and between cells (6, 19). In general, a hierarchy of functions has evolved for these different kinases such that growth and differentiation factor receptors receive extracellular signals and transmit these across the plasma membrane in the form of a tyrosine phosphorylation event. Downstream of the growth factor-receptor interaction is the activation by tyrosine phosphorylation of a number of cytoplasmic and nuclear serine/threonine kinases which are thought to be secondary effectors of the primary signal. There are, however, clear examples of variations to this theme. Nuclear isoforms of tyrosine kinases are known to exist (8, 43), and tyrosine phosphorylation of nuclear proteins has been demonstrated (3). The activin growth factor receptor appears to be the first example of a mammalian transmembrane kinase which phosphorylates serine/threonine rather than tyrosine residues (28).

We have been involved in the cloning of novel tyrosine kinases from embryonic and transformed cells by screening λgt11 expression libraries with antibodies to phosphotyrosine. While a number of kinases which exclusively phosphorylate tyrosine residues (23, 25, 26) have been identified in this way, we have also isolated cDNAs encoding serine/threonine/tyrosine (STY) kinases (4, 18).

Here we report the characterization of one of these kinases, the Esk kinase, and show that it exists in two isoforms, one of which appears to be a transmembrane molecule.

MATERIALS AND METHODS

Isolation of cDNA clones. Expression libraries were constructed from P19 embryonal carcinoma (EC) cells (18) as well as from CB7 and DP28-9 erythroleukemia cell lines (4) by using poly(A)$^+$ RNA and screened for active protein tyrosine kinases with use of an antiphosphotyrosine antibody. Essentially, 5×10^5 plaques were screened by infecting the bacterial strain Y1090 with recombinant bacteriophage and incubating the cells at 42°C for 4 to 6 h. The plates were then overlayed with filters presoaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 6 h at 37°C. Positive clones were identified by probing the filters with antiphosphotyrosine monoclonal antibody PY20 (ICN Biomedicals) and an alkaline phosphatase-conjugated anti-mouse secondary antibody.

Sequencing of cDNA clones. The *esk* cDNA inserts were subcloned into the *Eco*RI site of plasmid PTZ19R (Pharmacia). Nested deletions were generated by using an Exo-Mung deletion kit (Stratagene). Double-stranded DNA sequencing was carried out on the deletion mutants by using the dideoxy-chain termination method (36).

P19 EC cell maintenance and differentiation. P19 EC cells were cultured and differentiated as described previously (21, 29). Essentially, P19 cells were induced to differentiate into cells of the neural or muscle lineage by aggregating the cells in bacterial dishes in the presence of 0.5 μM retinoic acid or 1% dimethyl sulfoxide for 3 days and subsequently plating the cells onto coated tissue culture dishes to form monolayers in the absence of inducing drug.

RNA isolation and Northern (RNA) blot analysis. Total RNA was prepared from cells or tissues as described by Auffray and Rougeon (2). Poly(A)$^+$ RNA was selected by passage of total RNA through oligo(dT)-cellulose columns as described by Jacobson (20). Typically, aliquots of 5 μg of poly(A)$^+$ RNA were electrophoresed in a 1% agarose gel containing 19% formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS), 10 mM sodium acetate, and 1 mM EDTA, transferred to a Hybond N membrane (Amersham), and UV cross-linked for 2 min. Filters were prehybridized at 42°C for 12 to 16 h in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution (1% [wt/vol] Ficoll, 1% [wt/vol] polyvinylpyrrolidone, 1% [wt/vol] bovine serum albumin [BSA; fraction

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V])–5% dextran sulfate–0.1% sodium dodecyl sulfate (SDS)–0.5 mg of denatured salmon sperm DNA per ml. Hybridizations were performed by using a random-primed ^{32}P-labelled 1.5-kb fragment of the *esk-2* cDNA at 2×10^6 cpm/ml for 12 to 16 h at 42°C. Filters were washed in 0.1% SDS–0.1× SSC at room temperature and 42°C.

Bacterial expression of *esk* cDNA. A 1.5-kb fragment of the *esk-2* cDNA from the *Pvu*II site (position 901) to the *Eco*RI site (position 2417) was blunt ended with T4 DNA polymerase and subcloned in frame into the unique *Bam*HI site of the pET11b bacterial expression vector (Novagen). This places the cDNA insert under the control of the T7 promoter; expression is induced by supplying T7 RNA polymerase to the host cell. In *Escherichia coli* pLysS (Novagen), the polymerase gene is under *lac*UV5 control and induction is achieved by adding IPTG to the culture. Typically, pLysS bacteria expressing either the sense or antisense *esk* expression vector were grown to an optical density at 600 nm of 0.4 in M9CA minimal medium containing 100 μg of ampicillin per ml. The bacteria were induced for Esk protein production by addition of IPTG to a final concentration of 0.4 mM and incubation for an additional 2 h at 37°C.

Protein and phosphoamino acid analysis. (i) Immunoblotting. Whole cell extracts from *E. coli* pLysS induced to express *esk* were resolved on an SDS–7.5% polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell). Membranes were blocked with 20% serum in Tris-saline (140 mM NaCl, 10 mM Tris [pH 7.5]) for 1 h at room temperature and incubated with a 1:1,000 dilution of immunoglobulin G2bk (IgG2bk) antiphosphotyrosine antibody (UBI) in blocking buffer for 1 h at room temperature. Blots were washed three times in Tris-saline plus 0.05% Tween 20 and then incubated with an alkaline phosphatase-conjugated anti-mouse secondary antibody. Blots were washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

(ii) In vitro kinase assay. *E. coli* pLysS induced to express *esk* was lysed in 10 mM Tris (pH 7.5)–150 mM NaCl–5 mM EDTA–1% Triton X-100–2 mM NaF–2 mM sodium pyrophosphate–500 μM ammonium vanadate–200 μg of phenylmethylsulfonyl fluoride per ml–2 μg of aprotinin per ml–5 μg of leupeptin per ml and sonicated on ice three times for 20 s each time. Lysates were cleared by centrifugation, and supernatants were immunoprecipitated with either antiphosphotyrosine antibody PY20 (ICN Biomedicals) or IgG2bk (UBI). The immunoprecipitates were assayed for kinase activity in lysis buffer supplemented with 20 mM $MgCl_2$ and [γ-^{32}P]ATP or in buffers containing either 30 mM Tris (pH 7.5), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.1), or 20 mM 2[*N*-morpholino] ethanesulfonic acid (MES; pH 6.5), all supplemented with 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.2 mCi of [γ-^{32}P]ATP per ml for 30 min at 22°C. Myelin basic protein and acid-denatured enolase were added as exogenous substrates in some kinase assays. The reaction products were resolved on SDS–10% polyacrylamide gels, and the dried gels were exposed to Kodak XAR-5 X-ray film. Phosphoproteins were electroeluted for phosphoamino acid analysis by the method of Edwards et al. (9).

(iii) Renaturation kinase assay. The procedure was carried out essentially as described by Ferrell and Martin (11). Whole cell extracts from induced *E. coli* pLysS were resolved on SDS–10% polyacrylamide gels and transferred to Immobilon P membranes (Millipore) at 50 V for 90 min. Blotted proteins were denatured in 6 M guanidinium HCl–50 mM Tris–50 mM dithiothreitol–2 mM EDTA (pH 8.3) for 1 h at room temperature. Proteins were allowed to renature at 4°C for 12 to 16 h in 100 mM NaCl–50 mM Tris–2 mM dithiothreitol–2 mM EDTA–1% (wt/vol) BSA–0.1% (wt/vol) Nonidet P-40 (pH 7.5). Blots were blocked with 5% (wt/vol) BSA in 30 mM Tris (pH 7.5) for 1 h at room temperature. Kinase assays were performed by incubating the blots in 30 mM Tris (pH 7.5)–10 mM $MgCl_2$–2 mM $MnCl_2$–50 μCi of [γ-^{32}P]ATP per ml for 30 min at room temperature. Blots were washed twice in 30 mM Tris (pH 7.5), once in 30 mM Tris (pH 7.5)–0.05% Nonidet P-40, and once in 30 mM Tris (pH 7.5) (each wash was for 10 min). For phosphoamino acid analysis, regions of the membrane were excised and acid hydrolyzed in 6 N HCl for 75 min at 110°C as described by Kamps et al. (22).

Phosphatase treatment of Esk. Whole cell extracts of induced *E. coli* pLysS were resolved on SDS–10% polyacrylamide gels, and proteins were transferred to Immobilon P or nitrocellulose membranes as described above. Blots were blocked for 1 h with 5% (wt/vol) BSA in 30 mM Tris (pH 7.5) prior to treatment with potato acid phosphatase (PAP) (Sigma) or with the human phosphotyrosine phosphatase 1B (PTP1B) (a glutathione *S*-transferase [GST]-PTP1B fusion protein; generous gift of Ben Neel).

PAP treatment was an adaptation of the procedure described by Morrison et al. (31). Membrane strips prepared as described above were incubated with 80 μg of PAP per ml in a reaction buffer containing 20 mM Tris (pH 7.4), 5% glycerol, 0.05% Triton X-100, 2.5 mM $MgCl_2$, aprotinin (2 μg/ml), and leupeptin (5 μg/ml) for 1 h at 37°C with gentle shaking. An additional amount of PAP enzyme (80 μg/ml) was added, and the mixture was incubated for 1 h at 37°C. In some experiments, 1 mM ammonium vanadate was added to the buffer to inhibit phosphatase activity. The reaction was stopped by blocking the blots with 20% serum in Tris-saline for 1 h at room temperature. The blots were probed with an antiphosphotyrosine antibody as described above and detected by using an ^{125}I-labelled anti-mouse secondary antibody).

Isolation of the GST-PTP1B fusion protein. An overnight culture of *E. coli* DH5α(pGEX-PTP1B) was diluted 1:10 (with 100 μg of ampicillin per ml), grown for 1 h at 37°C, and induced for another hour by addition of IPTG to 1 mM. Bacteria were sonicated three times for 20 s each time in lysis buffer containing 10 mM imidazole-HCl (pH 7.2), 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, and 1% Triton X-100. Lysates were cleared by centrifugation, and the supernatants were incubated with 500 μl of 50% glutathione-coupled beads (Pharmacia) for 30 min at 4°C. The GST-PTP1B fusion was eluted twice with 1 ml of 5 mM reduced glutathione (Boehringer Mannheim) in phosphatase buffer. Glycerol was added to a final concentration of 33% (vol/vol), and aliquots of the enzyme (150 μl of PTP1B per ml of reaction buffer) were used in phosphatase experiments as described above.

STY kinase assay and phosphatase (GST-PTP1B) treatment. TrpE-STY fusion protein was prepared as described by Howell et al. (18). The STY fusion protein was immunoprecipitated with an anti-TrpE antibody (Oncogene Science) and allowed to autophosphorylate in the presence of [γ-^{32}P]ATP for 30 min at room temperature. The reaction products were resolved on SDS–10% polyacrylamide gels and transferred to an Immobilon P membrane, and individual strips were treated with PTP1B as described above.

Nucleotide sequence accession number. The GenBank accession number for the murine *esk-1* cDNA sequence is M86377.

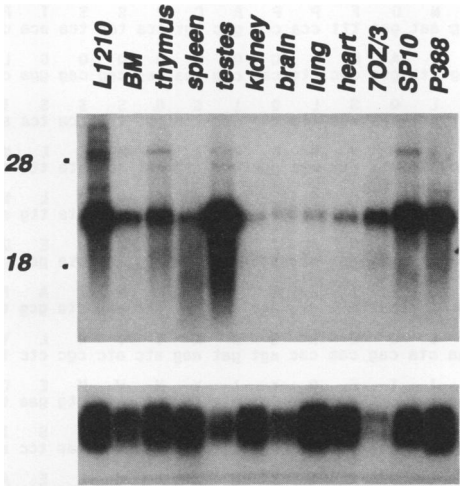

FIG. 1. Northern blot analysis of *esk* expression in adult mouse tissues. Five micrograms of poly(A)$^+$ RNA was isolated from the different adult mouse tissues and leukemic cell lines indicated above the lanes (BM, bone marrow). The blot was hybridized to a random-primed 1.5-kb cDNA probe derived from the *esk-2* cDNA. Positions of 18S and 28S rRNAs were determined by UV visualization of an acridine orange-stained marker lane. The lower panel represents the same Northern blot standardized with a β_2-microglobulin cDNA probe.

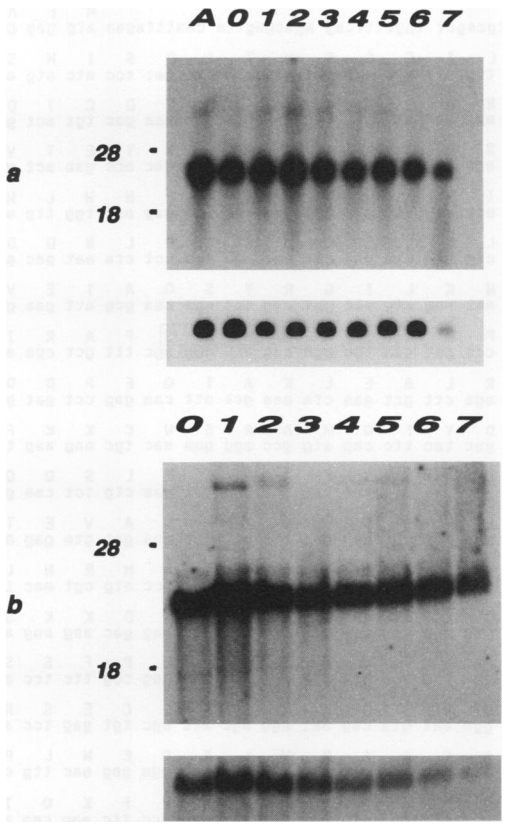

FIG. 2. Northern blot analysis of *esk* expression during P19 differentiation. Five micrograms of poly(A)$^+$ RNA was isolated from P19 cells induced to differentiate with retinoic acid (a) or with dimethyl sulfoxide (b). The blots were hybridized to the same 1.5-kb cDNA probe as in Fig. 1. Numbers above the lanes refer to days of differentiation; lane 0 refers to stem cells, and lane A in panel a refers to P19 cells aggregated in the absence of an inducing drug. mRNA levels were standardized by probing the blot with a cDNA to the phosphoglycerate kinase-1 gene. Positions of 18S and 28S rRNA are indicated.

RESULTS

Identification and expression of the *esk* gene product. mRNA isolated from P19 murine EC cells (21) was used to prepare a λgt11 cDNA library, which was then screened with antibodies to phosphotyrosine as described previously (4, 18, 26). A 2.97-kb cDNA isolated in this way was shown by sequence analysis to encode a novel protein kinase (see below) which we have named Esk (EC STY kinase).

To investigate the expression pattern of *esk*, Northern blot analysis was performed on a number of adult mouse tissues and cell lines. *esk* appears as a 3.0-kb mRNA (size estimated from rRNA markers) in cells which contain a significant subpopulation of proliferating cells (Fig. 1). Organs such as brain, heart, lung, and kidney had low amounts of *esk* mRNA, while testes, bone marrow, spleen, and thymus all contained higher levels of *esk* mRNA. The myeloma cell line SP10 and the B-cell leukemia lines L1210 and 70Z/3 also expressed a 3.0-kb *esk* mRNA. It appears, therefore, that *esk* transcripts are expressed in a limited spectrum of cell types with some capacity for proliferation. Following differentiation of P19 EC cells (21, 29) into the neuronal (using retinoic acid) or muscle (using dimethyl sulfoxide) lineage, *esk* mRNA levels remained constant (Fig. 2). During the time course of differentiation, there remains a significant proportion of proliferating cells (21, 28a), and we believe that it is these cells that express the *esk* transcripts.

Two distinct *esk* kinase isoforms are expressed in P19 cells. The nucleotide and deduced amino acid sequences of the 2.97-kb *esk* cDNA are presented in Fig. 3. The first methionine codon in the longest open reading frame is located at position 38, with a purine in the −3 position and a guanine residue in the +4 position, consistent with the consensus sequence of Kozak (24). No in-frame stop codons are found upstream of this ATG, preventing a formal assignment of this methionine as the site of translation initiation. Following this first methionine is an open reading frame encoding 857 amino acids ending in a TGA termination codon at nucleotide 2606. It is followed by a 362-nucleotide 3′ untranslated region containing a potential polyadenylation signal (AAT AAA at position 2901) which ends in 46 consecutive adenosine residues.

Analysis of the predicted translation product of the *esk* open reading frame revealed several interesting features. As expected, the Esk protein contains the 11 subdomains which are thought to collectively make up the catalytic region of protein kinases (16). Surprisingly, although the Esk kinase was cloned by virtue of its immunoreactivity with an antiphosphotyrosine antibody, comparison with the NBRF-PIR(r) data base by using the Lipman-Pearson algorithm (27) showed that its catalytic domain shows greatest similarity to serine/threonine kinases, including the yeast Snf1 serine/threonine kinase (7) (27% identity over a 402-amino-acid overlap), calcium/calmodulin-dependent protein kinase (40) (28% identity over 543 amino acids), and the human *pim* protein kinase (46) (25% over 329 amino acids). When individual subdomains were searched against the data base, an interesting homology was observed between Esk and a group of kinases that have STY kinase activity (4, 10, 18, 37). For convenience, we will refer to these bifunctional

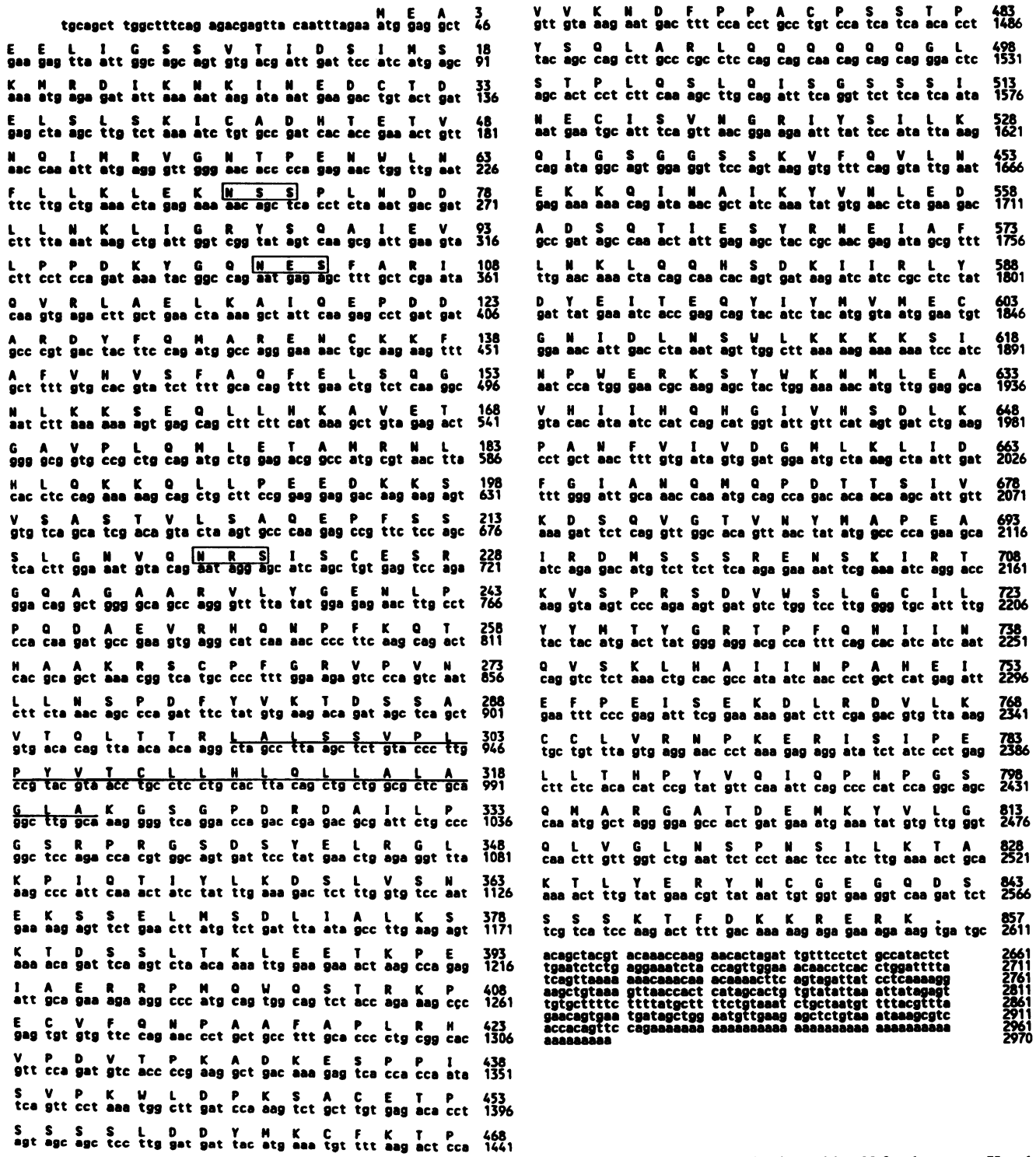

FIG. 3. Complete nucleotide and predicted amino acid sequences of the *esk-1* cDNA. The ATG codon in position 38 fits the proper Kozak consensus sequence and marks the initiation of the predicted protein. Potential glycosylation sites at amino acid positions 71, 102, and 219 are boxed, and the putative transmembrane domain between residues 296 and 321 is underlined. The Esk-2 predicted protein is identical to that shown here except that it lacks amino acid residues 296 to 321, the putative membrane-spanning domain.

kinases as members of the STY family of enzymes. It has been suggested by Seger et al. (37) that the homology in subdomain XI may be related to the novel specificity that these enzymes share. In this subdomain, Esk resembles the STY/Clk enzyme (4, 18) and Erk-1, a member of the *erk* kinase family (37) (Fig. 4). However, Wee1$^+$, a yeast STY kinase (10), displays little homology in this region, while Fus3 and Nim1$^+$, both yeast serine/threonine kinases (41,

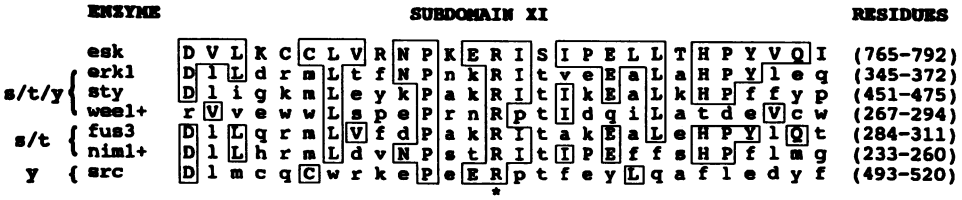

FIG. 4. Amino acid sequence comparison of subdomain XI. Sequences from subdomain XI of Esk, Erk-1, STY, Wee1$^+$, Fus3, Nim1$^+$, and Src were aligned without gaps or insertions. Amino acids identical to those of Esk are capitalized and boxed. The arginine marked by an asterisk is the conserved residue of this subdomain. The kinases have been grouped according to their presumed amino acid specificity: s/t/y; serine/threonine/tyrosine kinase; s/t, serine/threonine kinase; and y, tyrosine kinase.

35), show extensive homology to members of the STY family of kinases. These observations indicate that either the sequence homology in subdomain XI is insufficient to assign serine/threonine/tyrosine specificity to a kinase or, alternatively, Fus3 and Nim1$^+$ may prove to be new members of the STY family.

The noncatalytic domain of Esk contains a very hydrophobic stretch of 26 amino acids followed by a lysine residue between residues 296 and 321 (Fig. 3, underlined amino acids). This configuration of amino acids is reminiscent of the transmembrane domains of receptor molecules (44) and would partition Esk into a 295-amino-acid extracellular domain and a 536-amino-acid cytoplasmic region. The putative extracellular portion of Esk contains three potential sites of N-linked glycosylation (NXS or NXT) at positions 71, 102, and 219. Taken together, these observations suggest that this isoform of the Esk kinase, termed the Esk-1 kinase, is a glycosylated transmembrane kinase. Inspection of the predicted amino terminus of Esk-1 did not reveal a convincing cleavable signal sequence peptide. There are several examples of integral membrane proteins lacking amino-terminal signal peptides, perhaps most relevantly the transmembrane protein tyrosine kinase Ltk (5).

Two additional *esk* clones, a 2.85-kb cDNA isolated from a mouse erythroleukemia cell line library (4, 25) and a 2.0-kb cDNA isolated from the P19 library, were sequenced and compared with the original *esk-1* cDNA. While the catalytic domains of all three cDNAs were identical, there was an interesting and discrete difference in the predicted noncatalytic regions of the cDNAs. Both *esk-2* cDNAs lack the amino acids which we believe form the membrane-spanning domain of Esk-1. The poly(A) tract at the 3′ end of the larger *esk-2* cDNA is 39 nucleotides shorter than the poly(A) tail of *esk-1*; however, the 5′ ends of the two cDNAs are identical. Since these clones were isolated from two separate libraries, it seems likely either that this region represents the authentic 5′ end of the *esk* mRNA or there exists a strong stop for reverse transcriptase at this position preventing the isolation of full-length cDNA clones. In either case, it appears that two distinct Esk isoforms are expressed in P19 EC cells, one of which is likely a transmembrane molecule.

Esk has kinase activity in vitro. The *esk* cDNAs were isolated by virtue of the fact that their gene products were recognized by an antiphosphotyrosine antibody. Paradoxically, the predicted amino acid sequence of the Esk catalytic domain shows greatest similarity to sequences of serine/threonine kinases. Similar observations have been made for several members of the STY family of protein kinases (4, 18, 37, 39). To determine the specificity of the Esk kinase, the catalytic domain was subcloned into a pET bacterial expression vector under the control of the T7 promoter as described in Materials and Methods. Western blot analysis of bacterial extracts containing the Esk kinase with antiphosphotyrosine antibodies revealed an immunoreactive band of approximately 75 kDa (Fig. 5a). Cells transfected with the expression vector containing *esk* in the antisense orientation did not contain any immunoreactive proteins. This immunoreactivity was specific for phosphotyrosine, since phosphoserine and phosphothreonine did not compete for antibody binding, whereas phosphotyrosine completely abolished Esk immunoreactivity with the antiphosphotyrosine antibody (data not shown). When the same antibody was used for immunoprecipitation followed by an in vitro kinase assay, a 75-kDa protein was again identified, while control cells were negative (Fig. 5b). To rule out the possibility that Esk phosphorylation in these immunoprecipitates resulted from a coprecipitating bacterial kinase, in situ kinase reactions were performed. Essentially, Esk bacterial extracts were resolved on SDS-gels, transferred to Immobilon membranes, renatured and then incubated with [γ-^{32}P]ATP as described previously (11). As with the immunoblots and immunoprecipitates, a 75-kDa band was identified in Esk bacterial extracts but was absent in extracts prepared from bacteria expressing the antisense expression vector (Fig. 5c). Phosphoamino acid analysis was performed on Esk protein

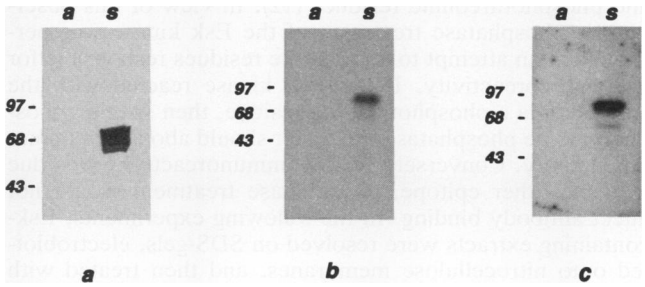

FIG. 5. Kinase activity of Esk in vitro. (a) Western analysis of Esk. Lysates of *E. coli* pLysS expressing *esk* in the antisense (a) or sense (s) orientation were run on SDS–7.5% polyacrylamide gels and transferred to nitrocellulose. The membrane was probed with the IgG2bK antiphosphotyrosine antibody (UBI) and detected with an alkaline phosphatase-conjugated anti-mouse secondary antibody. (b) In vitro kinase assay. The Esk kinase was immunoprecipitated from *E. coli* pLysS extracts (either sense or antisense) by using an antiphosphotyrosine antibody and assayed for kinase activity by adding [γ-^{32}P]ATP to the immunoprecipitates. The products were resolved on SDS–10% polyacrylamide gels. (c) Renaturation kinase assay. Lysates of *E. coli* pLysS expressing *esk* in either orientation were run on SDS–10% polyacrylamide gels and transferred to Immobilon P membranes. The proteins were denatured and renatured as described in Materials and Methods and assayed for kinase activity by adding [γ-^{32}P]ATP to the membrane. Sizes are indicated in kilodaltons.

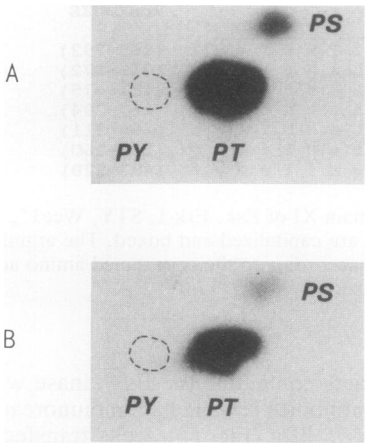

FIG. 6. Phosphoamino acid analysis of labelled Esk kinase. Labelled Esk kinase derived from an in vitro kinase assay (A) or from a renaturation kinase assay (B) was acid hydrolyzed, and phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis. The positions of the phosphoamino acid standards were identified by ninhydrin staining. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

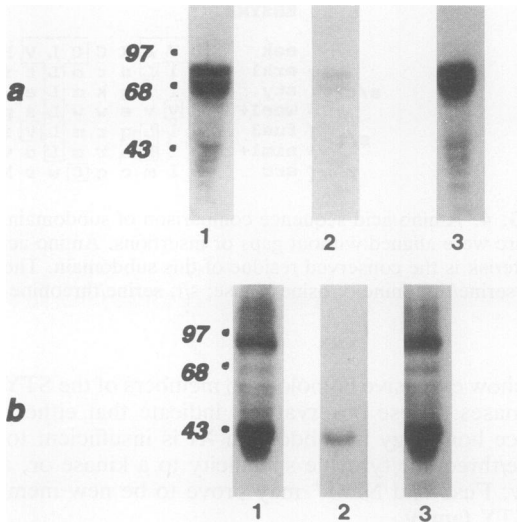

FIG. 7. Sensitivity of Esk immunoreactivity to a phosphotyrosine-specific phosphatase. (a) Esk bacterial extracts were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose. GST-PTP1B fusion protein was used to phosphatase treat the individual membrane strips. Lanes: 1, untreated; 2, phosphatase treated; 3, phosphatase treated in the presence of 1 mM ammonium vanadate. Blots were probed with an antiphosphotyrosine antibody and ^{125}I-labelled secondary antibody and then subjected to autoradiography. (b) Bacterial extracts expressing a TrpE-STY fusion protein (18) were treated exactly as for panel A as a control for GST-PTP1B activity. The TrpE-STY fusion has a molecular size of 85 kDa, and a breakdown product runs at 43 kDa. Lanes are as described for panel A. Sizes are indicated in kilodaltons.

labelled in immunoprecipitates or following renaturation, and only phosphoserine and phosphothreonine residues were identified (Fig. 6). Although the Esk protein is immunoreactive with antiphosphotyrosine antibodies, only phosphoserine and phosphothreonine residues were labelled in these in vitro conditions.

Phosphatase treatment of Esk. Since the Esk kinase was immunoreactive with antiphosphotyrosine antibodies yet displayed no in vitro tyrosine kinase activity, we were concerned about the specificity of the antiphosphotyrosine antibody. Previously, others had reported that certain monoclonal antiphosphotyrosine antibodies cross-react with 5′-mononucleotides and with phosphohistidine, phosphoserine, and phosphothreonine residues (12). In view of this observation, phosphatase treatment of the Esk kinase was performed in an attempt to identify the residues responsible for its immunoreactivity. If the Esk kinase reacted with the antibody via a phosphotyrosine residue, then specific phosphotyrosine phosphatase treatment should abolish its immunoreactivity. Conversely, if Esk immunoreactivity was due to some other epitope, phosphatase treatment would not affect antibody binding. In the following experiments, Esk-containing extracts were resolved on SDS-gels, electroblotted onto nitrocellulose membranes, and then treated with either PAP or the human PTP1B (expressed as a GST fusion protein). Preparations of PAP are known to have reactivity toward phosphoserine, phosphothreonine, and phosphotyrosine, while PTP1B preferentially dephosphorylates phosphotyrosine residues (42). The filters were then reacted with antibodies to phosphotyrosine and ^{125}I-labelled secondary antibodies. As shown in Fig. 7a, treatment of Esk with PTP1B eliminated its immunoreactivity, as did treatment with PAP (data not shown). Filters incubated with PAP or PTP1B in the presence of vanadate, a phosphatase inhibitor, retained the epitope recognized by the antiphosphotyrosine antibody, confirming that the loss of immunoreactivity was due to phosphatase activity rather than a contaminating protease. To verify that our preparation of PTP1B was specific for phosphotyrosine, it was tested on a substrate which is known to contain all three phosphorylated hydroxy amino acids. For these experiments, we used STY/Clk, a kinase which we have shown autophosphorylates on serine, threonine, and tyrosine residues (4, 18). Like Esk, the STY kinase lost its immunoreactivity with the antiphosphotyrosine antibody when treated with PTP1B (Fig. 7b). To further define the specificity of the phosphatase, the TrpE-STY fusion protein was immunoprecipitated and incubated with [γ-^{32}P]ATP in a kinase assay as previously described (18). The products of the kinase reaction were run on SDS-gels, transferred to Immobilon, and treated with PTP1B. The samples were acid hydrolyzed, and phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis. From Fig. 8, it is clear that the PTP1B prepa-

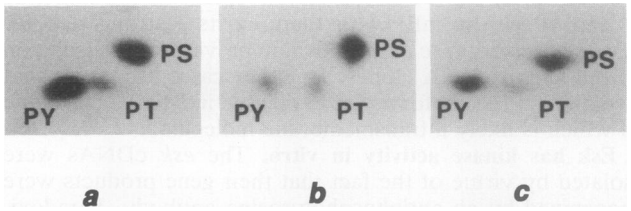

FIG. 8. Phosphotyrosine specificity of the GST-PTP1B phosphatase. The STY kinase was subjected to an in vitro kinase assay, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon. Membrane strips were treated with GST-PTP1B or with GST-PTP1B in the presence of 1 mM ammonium vanadate. Phosphoamino acids of untreated STY (a), of STY treated with phosphatase (b), of STY treated with phosphatase in the presence of vanadate (c) were analyzed by two-dimensional thin-layer electrophoresis.

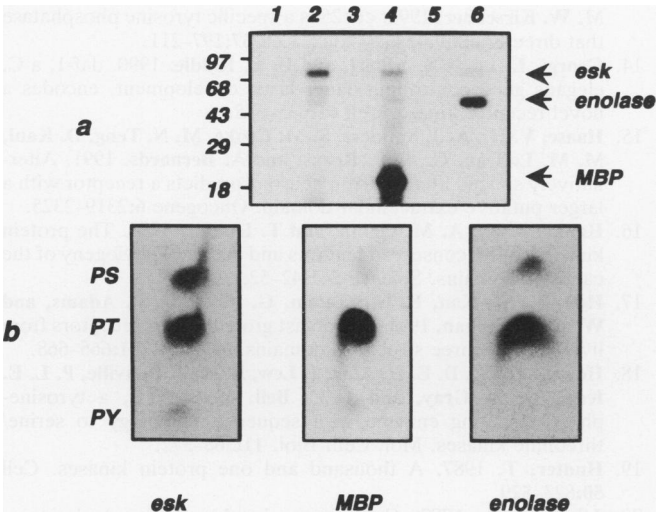

FIG. 9. Serine, threonine, and tyrosine kinase activities of the Esk kinase. (a) In vitro kinase assay. The Esk kinase was immunoprecipitated from *E. coli* pLysS extracts (either sense [lanes 2, 4, and 6] or antisense [lanes 1, 3, and 5] orientation) by using an antiphosphotyrosine antibody and assayed for kinase activity by adding [γ-^{32}P]ATP to the immunoprecipitates. Myelin basic protein (MBP) was added to the immunoprecipitates in lanes 3 and 4, while acid-denatured enolase was added in the assays in lanes 5 and 6. Sizes are indicated in kilodaltons. (b) Phosphoamino acids were generated from the labelled protein bands in lanes 2, 4, and 6 and resolved by one-dimensional thin-layer electrophoresis (as described in Materials and Methods). Positions of the phosphoamino acid standards were identified by ninhydrin staining. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

ration used in these experiments removes phosphate groups from tyrosine but not serine or threonine residues. These results confirm that Esk contains a phosphotyrosine residue(s) which was not detected in our initial in vitro or renaturation kinase assays.

Esk has serine, threonine, and tyrosine kinase activities. In the preceding experiments, kinase reactions were performed in a lysis buffer containing salt and detergents supplemented with magnesium (see Materials and Methods). We therefore attempted to define the buffer conditions that might reveal Esk tyrosine kinase activity in vitro. Kinase assays were carried out in a series of buffers (HEPES, MES, and Tris) at different pH conditions supplemented with magnesium and/or manganese. We found that the Esk kinase had optimum activity in buffers ranging between pH 6.5 and 7.0 containing 2 mM manganese. Under these conditions, Esk was able to autophosphorylate and phosphorylate exogenous substrates on serine, threonine, and tyrosine residues. The results of one such experiment are shown in Fig. 9. In Fig. 9a, it is evident that Esk not only can autophosphorylate (lane 2) but also has activity toward myelin basic protein (lane 4) and enolase (lane 6). Immunoprecipitates from extracts of bacteria expressing antisense *esk* mRNA lacked kinase activity (lanes 1, 3, and 5). Amino acid analysis of the phosphorylated products (Fig. 9B) revealed that threonine was the preferred target in all three substrates, although phosphotyrosine and phosphoserine were also present. Taken together, the results obtained from Western blotting, the phosphatase experiments, and the in vitro kinase assays clearly demonstrate that the Esk kinase has serine, threonine, and tyrosine kinase activities and is therefore a novel member of the STY family of protein kinases.

DISCUSSION

Several protein kinases appear to exist in multiple isoforms encoded by distinct mRNA species. In this regard, Esk represents a novel departure from previously identified alternatively spliced kinases in that Esk is the first example of a kinase which may exist both as a transmembrane molecule and as a soluble catalytically active enzyme. Indeed, most alternative splicing events in receptor protein kinases generate molecules with variant ligand binding affinities or specificities as for the fibroblast growth factor, Ltk, and activin receptors (1, 15, 17). Alternatively, these splicing events have also been shown to generate a truncated receptor in the case of the neurogenic TrkB kinase or for the epidermal growth factor receptor, truncated and secreted (30, 32).

The cloned *esk* cDNAs do not encode an amino-terminal signal peptide, and it remains possible that the putative transmembrane domain is itself the signal peptide (44). Indeed, the signals which determine the subcellular localization of proteins are incompletely understood and are likely encoded in the three-dimensional structure of the protein (44). Furthermore, there appears to be a hierarchy of subcellular localization signals (34), and thus insertion of a transmembrane domain in the Esk-1 protein may override the localization signals shared between Esk-1 and Esk-2.

To date, only one mammalian transmembrane kinase, the activin receptor, is believed to have serine/threonine instead of tyrosine kinase activity (28). Despite the high degree of homology between Esk and members of the serine/threonine kinase family, and we have found very little sequence homology between Esk and the activin receptor. Two other transmembrane serine/threonine kinases, Daf-1 from *Caenorhabditis elegans* and Zmpk1 from maize, also have very little homology to Esk (14, 45). Through the recent cloning of these novel serine/threonine receptor kinases (14, 28, 45) and the Esk-1 STY receptor kinase, it has become clear that cell proliferation and differentiation signals mediated by receptor kinases are not restricted to tyrosine phosphorylation events.

Amino acid sequence comparisons revealed that Esk shares greatest homology with members of the serine/threonine family of protein kinases, and in particular with the catalytic domains of the yeast kinases Snf1 and Nim1$^+$ (7, 35). Seger et al. (37) have recently reported that conserved amino acid motifs exist in subdomain XI of a family of kinases with the capacity to phosphorylate serine, threonine, and tyrosine residues. Since this region is generally not well conserved among kinases, it was suggested that it may play some role in determining substrate specificity. Both Esk and Nim1$^+$ show considerable homology to the STY kinases in this region; however, they have a more pronounced homology to each other. The similarity of this domain in the Esk and Nim1$^+$ kinases may reflect conserved biochemical activities for these two enzymes.

Threonine and tyrosine phosphorylation events are important in regulating the activity of several signal transduction components. The activities of the epidermal growth factor receptor kinase, the cell cycle-regulating kinase p34^{cdc2}, and the MAP kinases have all been shown to be regulated through threonine and tyrosine phosphorylation events (13, 33, 38). Since Esk appears to exist as both a transmembrane and soluble catalytically active enzyme, its threonine and

tyrosine kinase activities could be involved in modulating different levels of signal transduction pathways.

The Esk kinase has a restricted pattern of expression in the adult mouse, found predominantly in tissues which have a significant stem cell component. We are currently determining the spatial and temporal expression patterns of the different Esk kinase isoforms. Defining the specific distribution of the Esk-1 kinase will aid in the identification of its ligand.

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