A Widely Expressed Transmembrane Serine/Threonine Kinase That Does Not Bind Activin, Inhibin, Transforming Growth Factor β, or Bone Morphogenetic Factor

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Molecular cloning of complementary DNAs (cDNA) whose expression products bind activin and transforming growth factor β (TGF-β1 and -β2) suggests that transmembrane serine/threonine kinases constitute a new class of signaling molecules. A human liver cell cDNA which codes for a new serine/threonine kinase receptor (SKR1) was identified using degenerate oligonucleotide primers complementary to coding sequence for mouse activin and Caenorhabditis elegans daf-1 serine/threonine kinase receptor subdomains VI and VIII in the polymerase chain reaction. The deduced 509-amino acid product consisted of a cysteine-rich extracellular domain and a cytoplasmic serine/threonine kinase domain which are 10–20 and 40% homologous to the respective domains in the activin and transforming growth factor β receptor kinases. Cells overexpressing SKR1 exhibited no increase in binding of activin, inhibin, TGF-β1, TGF-β2, or bone morphogenetic factor type 2B. Except for its absence in bone and spleen, SKR1 exhibits a tissue expression pattern similar to the TGF-β receptor II gene. Similarly, SKR1 is expressed in normal parenchymal cells, endothelial cells, fibroblasts, and tumor-derived epithelial cells. The expression pattern and lack of binding to prototypic members of the TGF-β1–5 branch of the TGF-β superfamily suggests that SKR1 is potentially a receptor for a new member of the TGF-β branch of the ligand superfamily.

Recent molecular cloning of cDNAs coding for proteins that bind activin and transforming growth factor β types 1 and 2 (TGF-β1 and -β2) suggest that receptors for the TGF-β superfamily of regulatory polypeptides are transmembrane serine (Ser)/threonine (Thr) protein kinases (1–5). Although the 120–160-residue cysteine-rich extracellular domains of the two receptors exhibit limited homology, both have conserved clusters of residues in the intracellular domain that define subdomains of the Ser/Thr kinase superfamily (6, 7). To characterize Ser/Thr kinase receptors in human hematopoietic cells, we exploited the polymerase chain reaction (PCR) to generate cDNAs to kinase subdomains VI and VIII in the activin receptor kinase and a homolog from Caenorhabditis elegans (daf-1) (8). In addition to cDNA fragments coding for the human homolog of mouse activin receptor IIb (ActRIIB) (1–4) and the human ActRII (9), we identified a novel cDNA (SKR1) that is homologous to, but distinct from, the activin receptor isoforms (1–4) and the TGF-β1 and -β2 receptor II kinases (TGFβRII) (5). Here we report the deduced structure of SKR1 and show that the product of SKR1 cDNA binds neither activin, inhibin, TGF-β1 and -β2 nor bone morphogenetic factor type 2B (BMP-2B). SKR1, which is widely expressed in both normal and tumor tissues and cells similar to the TGFβRII, may be a receptor for a novel member of the TGF-β branch of the superfamily of ligands.

EXPERIMENTAL PROCEDURES

RNA Extraction and Analysis—RNA isolation and Northern hybridization analysis were performed as described previously (10, 11) except that the hybridization for SKR1 was carried out in 40% formamide, 6 × SSC, 0.1% SDS, 100 µg/ml salmon sperm DNA, and 0.1% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. Complementary 32P-labeled cDNA probes were a 1.3-kb PstI/XbaI fragment, a 0.5-kb XbpI fragment coding for the extracellular domains of SKR1, TGFβRII, and ActRII, respectively.

PCR and Analysis of Products—Random-primed cDNA was synthesized from poly(A)+ RNA prepared from the human hepatoma cell, HepG2, and subjected to 40 cycles of amplification for 1 min at 94 °C, 2 min at 45 °C, and 3 min at 72 °C with primer mixtures (5'-GGAATTCATC/C/CTCCG/C/GAAGC/CT/AT/C/GAA/GA/GAT/G/ C(G)/C/AA/AA/3') and (5'-CCTCGAG/C/TCCG/G/TCNGGGCGCAG/AG/G/ TANC-3') in the PCR where n = all four nucleotides. DNA fragments were separated on agarose gels and tested for hybridization with mouse ActIII cDNA, a gift from Dr. W. Vale (Salk Institute, La Jolla, CA). The positive 180-base pair (bp) band was excised from the gel, digested with EcoRI/Xhol, and cDNA clones were prepared in bacterial SK vector (Stratagene, La Jolla, CA). Nucleotide sequence of activin and SKR1 cDNA clones was determined as described (11).

Deduction of the Full-length SKR1 Sequence—Ten groups of 10° clones from a HepG2 cDNA phage library (CloneTech, Inc., San Diego, CA) were screened by PCR amplification of a specific 101-bp SKR1 sequence between the coding sequences for kinase subdomains I on the GeneBankTM/EMBL Data Bank with accession number(s) L09291.

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The abbreviations used are: TGF-β, transforming growth factor type β; cDNA, complementary DNA; SKR1, serine/threonine kinase receptor type 1; PCR, polymerase chain reaction; TGFβRII, transforming growth factor β receptor type II; ActRII, activin receptor type II; BMP, bone morphogenic factor; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s); bp, base pair(s).
annulling temperaing was 56 °C. Serial dilution and sequential screening of positive groups yielded one 1.7-kb cDNA which coded for SKR1 beginning at His-320 and extended through a long open reading frame followed by a poly(A) tract. Application of rapid amplification of cDNA ends (RACE)-PCR (12) using the 180-bp cDNA across subdomains VI and VI11 (Fig. A) as a primer yielded cDNAs beginning at coding sequence for residue Gly-77 in the extracellular domain of SKR1. A sense primer based on the 5' most sequence of the above cDNA (5'GGGAAAGAT-3') was used to further screen the HepG2 library as described above and resulted in a 1.8-kb cDNA that contained the complete amino acid sequence of SKR1. The complete nucleotide sequence of SKR1 (GenBank M85079) (5) and ActRII (GenBank X63123) (9) sequences are in inverse print. Extracellular cysteines (solid boxes), one potential N-linked glycosylation sites (open box), the transmembrane domain (solid bar), and conserved Ser/Thr residues (open circles) are indicated. Kinase subdomains VI and VIII complementary to primers A and B (Fig. 1A) are overlined. Conserved potential tyrosine phosphorylation sites are indicated by closed circles.

Expression of SKR1 in Baculoviral-infected Sf9 Insect Cells—A 2.4-kb coding for full-length SKR1 was cloned into the EcoRI site of the transfer vector pVL1393 (Invitrogen, San Diego, CA). Recombinant virus was generated by co-infection of wild-type baculoviral DNA (0.5 μg) containing a lethal deletion (BaculoGold DNA, Pharmingen, San Diego, CA) and 2 μg of the SKR1-pVL1393 construct. Superinfective virus was generated by co-infection of wild-type baculoviral DNA (0.5 μg) containing a lethal deletion (BaculoGold DNA, Pharmingen, San Diego, CA) and 2 μg of the SKR1-pVL1393 construct.

Identification and Characterization of SKR1—To identify activin-related and potentially novel Ser/Thr kinase receptor cDNAs, we designed degenerate sense (1024 sequences) and antisense (256 sequences) oligonucleotides complementary to the amino acid sequences of kinase subdomains VI and VIII (6, 7) which are conserved between the mouse ActRII gene (1) and the C. elegans daf-1 gene (8) (Fig. 1A). Oligonucleotide primers were used in the PCR with template cDNA from a mammalian expression vector pVL1393 (Invitrogen, San Diego, CA). Recombinant viral plaques were identified by a combination of visual screening and Southern blot hybridization. Sf9 cells were prepared, infected with virus (13), and tested for binding of 125I-labeled TGF-β1 (3.2 × 10^6 cpn, 1.3 ng/ml) and BMP-2B (2.3 × 10^6 cpn, 10.7 ng/ml). Cells were washed three times with binding buffer, extracted with 1% Triton X-100 in phosphate-buffered saline, and the extract was counted using a γ-counter.

Immunoechemical Analysis of Infected Sf9 Cells for Expression of SKR1—Immunoechemical analysis were carried out as described previously (17) except that the presence of cell surface SKR1 antigen was analyzed with a rabbit antiserum prepared against the synthetic peptide sequence, EDEKPKVNPKLYMCV, from the NH2 terminus of SKR1. SKR1 antigen was analyzed with a rabbit antiserum prepared against the synthetic peptide sequence, EDEKPKVNPKLYMCV, from the NH2 terminus of SKR1.

RESULTS AND DISCUSSION

Identification and Characterization of SKR1—To identify activin-related and potentially novel Ser/Thr kinase receptor cDNAs, we designed degenerate sense (1024 sequences) and antisense (256 sequences) oligonucleotides complementary to the amino acid sequences of kinase subdomains VI and VIII (6, 7), which are conserved between the mouse ActRII gene (1) and the C. elegans daf-1 gene (8) (Fig. 1A). Oligonucleotide primers were used in the PCR with template cDNA from a mammalian expression vector pVL1393 (Invitrogen, San Diego, CA). Recombinant viral plaques were identified by a combination of visual screening and Southern blot hybridization. Sf9 cells were prepared, infected with virus (13), and tested for binding of 125I-labeled TGF-β1 (3.2 × 10^6 cpn, 1.3 ng/ml) and BMP-2B (2.3 × 10^6 cpn, 10.7 ng/ml). Cells were washed three times with binding buffer, extracted with 1% Triton X-100 in phosphate-buffered saline, and the extract was counted using a γ-counter.

Immunoechemical Analysis of Infected Sf9 Cells for Expression of SKR1 Surface Antigen—Immunoechemical analysis were carried out as described previously (17) except that the presence of cell surface SKR1 antigen was analyzed with a rabbit antiserum prepared against the synthetic peptide sequence, EDEKPKVNPKLYMCV, from the NH2 terminus of SKR1. SKR1 antigen was analyzed with a rabbit antiserum prepared against the synthetic peptide sequence, EDEKPKVNPKLYMCV, from the NH2 terminus of SKR1.

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FIG. 2. Lack of binding of activin, TGF-β1, and BMP-2B to SKR1. COS cells were transiently transfected (11) with mammalian expression plasmids bearing ActRII, SKR1, or TGFβRII in sense (S) or antisense (A) orientations as indicated. Transfected cells (S or A) or cells (10^6) exposed to the transfection procedure alone (M) attached to 25-cm^2 culture dishes were then incubated with labeled ligand in absence (–) of presence (+) of 1000-fold excess of unlabeled ligand in 1.5 ml of binding medium (cell culture nutrient medium plus 1 mg/ml bovine serum albumin), the cells were then extracted with detergent, and the extract was counted and expressed as a percent of labeled ligand with sense and antisense SKR1 cDNA, respectively. Cells bearing labeled ligands were incubated with homobifunctional covalent cross-linked bound cells transfected with sense TGFBRII cDNA and mock-transfected cells, respectively; in the assays. 3T3 mouse fibroblasts were used as a positive control for BMP-2B binding assays. Inset of A: lanes 1 and 2, [125I]-TGF-β1 bound cells transfected with sense TGFBRII cDNA and mock-transfected cells, respectively; lanes 3 and 4, cells transfected with SKR1 antisense and sense cDNA, respectively. Inset of C: lane 1, mouse 3T3 cells bearing labeled BMP-2B; lanes 2 and 3, COS cells transfected with sense and antisense SKR1 cDNA, respectively. Cells bearing labeled ligands were incubated with homobifunctional covalent cross-linked agent, disuccinimidyl suberate, and the detergent extract was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (11).

FIG. 3. Expression of SKR1 in baculoviral-infected Sf9 insect cells. Sf9 cells were prepared, infected with virus (13) and uninfected (–) and infected (+) cells were tested for [125I]-labeled TGF-β1 (A) and BMP-2B (B) binding as described in Fig. 2. C, cell surface antigen. Surface antigen was analyzed under the same conditions and on cells from the same transfections described in A and B using antisera against the NH2 terminus of SKR1.

Human hepatoma cell line HepG2 (11). Hybridization of PCR-generated fragments at low stringency with mouse ActRII cDNA indicated the presence of a band at about 180 bp, consistent with the presence of human activin or related cDNA fragments. The 180-bp band was excised, cloned, and 37 clones subjected to sequence analysis. Twenty cDNA clones exhibited nucleotide sequences for amino acid residue landmarks for Ser/Thr kinase subdomain VII which is between subdomains VI and VIII (1, 6, 7). Six clones exhibited nucleotide sequences identical to human ActRII (9), amino acid sequences deduced from four cDNAs were identical to mouse ActRIB (2, 3), and the rest coded for a Ser/Thr kinase (SKR1) that was significantly different from cDNAs coding for human Ser/Thr receptor kinases ActRII and TGFβRII (5, 9). Similar to the C. elegans daf-1 gene (8), the SKR1 cDNA coded for a DLG sequence instead of the DFG tripeptide sequence in human ActRII and TGFβRII which is highly conserved and defines subdomain VII throughout the Ser/Thr kinase superfamily (6, 7). The complete nucleotide sequence of SKR1 was determined by isolation of cDNA clones using a combination of PCR-based screening of a HepG2 cDNA phage library and RACE-PCR. The complete nucleotide sequence of the SKR1 cDNA extended for 3000 nucleotides and exhibited a single long open reading frame of 1527 nucleotides which encodes a putative 58-kDa primary translation product of 509 amino acids (Fig. 1B). The predicted product of the SKR1 gene meets the criteria for a transmembrane Ser/Thr kinase receptor. The first 21 amino acids are hydrophobic and likely serve as a signal sequence for membrane transport. Hydropathy analysis revealed an additional stretch of 23 hydrophobic residues followed by a cluster of basic amino acids that probably serve as the transmembrane domain and stop-transfer signal. The extracellular domain includes 10 cysteines and one potential N-linked glycosylation site (Fig. 1B). Except for a similarity in spatial distribution of the cysteine residues, SKR1 exhibited only a 12 and 20% amino acid sequence homology, respectively, with human ActRII and TGFβRII Ser/Thr receptors in the extracellular domain (Fig. 1B). Although there is less than 5% homology in the juxtamembrane sequences, the intracellular domain of SKR1, which has consensus ATP-binding and a kinase domain as well as a COOH-terminal tail, exhibited a 40% homology with both human ActRII and TGFβRII receptors. Although there is yet no context consensus sequences for Ser/Thr kinase receptor substrate phosphorylation sites, SKR1 exhibits seven Ser/Thr sites in the intracellular domain that are conserved in all three Ser/Thr receptor kinases that may be potential autophosphorylation sites (Fig. 1B). Noteworthy
FIG. 4. Expression of SKR1 mRNA in various cells and rat tissues. The indicated cell types were from the following sources: HepG2, human hepatoma (ATCCHB8065); Hep3B, human hepatoma (ATCCHB8064); A431, human epidermoid carcinoma (ATCC-CRL1555); HUV-EC, human umbilical vein endothelium (18, 19); HPF, human foreskin (fibroblast); HK, human foreskin keratinocytes; rat hepatocytes (20, 21); mouse 3T3 fibroblasts; DT, rat Dunning tumor R3327PAP (nonmalignant epithelial) (10); AT3, rat Dunning tumor R3327AT3 (malignant epithelial) (10). The HK lane contained 20 μg total RNA and other lanes contained 2.5 μg of poly(A+)RNA hybridized as described (10) with 125I-labeled cDNA probes complementary to the extracellular domains of the indicated genes.

is the conservation in flanking sequences around the conserved Thr/Ser (Thr-487 in SKR1) at the beginning of the COOH-terminal tail of the three genes. Potential tyrosine phosphorylation sites in SKR1 are conserved in the TGFβRII gene (Tyr-294) and both the TGFβRII and ActRII genes (Tyr-427).

Ligand Binding to Recombinant SKR1—To determine whether SKR1 was an isoform of the activin or TGFβRII Ser/Thr kinase receptors, we transfected monkey kidney cells (COS) with a mammalian expression vector bearing an SKR1 cDNA and tested for an increase in 125I-labeled activin and TGF-β1 binding. In contrast to cells transfected with vector bearing ActRII and TGFβRII cDNAs, cells transfected with SKR1 cDNA exhibited no increase in binding of the two ligands (Fig. 2, A and B), although analysis of mRNA confirmed that SKR1 mRNA was overexpressed. Separate experiments in which labeled inhibin and TGF-β2 was substituted for activin and TGF-β1 revealed no increase in ligand binding to cells transfected with SKR1 cDNA. In addition no increase in 125I-labeled bone morphogenetic factor type 2B (BMP-2B) could be detected in SKR1-transfected cells (Fig. 2C) relative to mouse fibroblast cells which exhibit BMP-2B binding sites (16). The lack of binding of TGF-β1 and BMP-2B to insect cells infected with recombinant baculovirus bearing the SKR1 cDNA and which express cell surface SKR1 antigen confirmed the negative binding results in transiently transfected mammalian cells (Fig. 3).

Expression of SKR1 Tissues and Cells—The expression of SKR1 in cells and tissues was compared with that of the ActRII and TGFβRII receptor kinases. Northern hybridization using cDNAs coding for the extracellular domain of the three genes was performed to avoid potential cross-hybridization between homologous intracellular domains (Fig. 4). Human cells exhibited a major 3.6 and two minor 5.8- and 8.6-kb mRNAs, whereas rat and mouse cells and tissues exhibited a major species at 4.2 kb. The tissue expression pattern of SKR1 is more similar to TGFβRII than that of ActRII. SKR1 was most abundant in heart, liver, intestine, and kidney, low in brain and lung, and undetectable in spleen and bone. In contrast, TGFβRII mRNA was undetectable in brain, higher in lung and liver, lower in heart and intestine, but detectable in spleen and bone. Similar to the TGFβRII gene, SKR1 is expressed in both normal parenchymal cells (skin keratinocytes and hepatocytes) and endothelial cells and fibroblasts (Fig. 4). Of the cells examined, the hepatoma cells (HepG2) from which SKR1 was cloned and endothelial cells exhibited the highest levels of expression of both SKR1 and TGFβRII.

Our results suggest that SKR1 is a receptor for an unidentified member of the TGF-β ligand superfamily (1–5). SKR1 may be a specific receptor for one or more of the cloned, but less well characterized members or a completely new member, of the superfamily (1–5). The lack of binding to a prototypic member (BMP-2B also called BMP-4) of the BMP-2, -3, and -4 branch of the superfamily and absence of SKR1 in bone suggests that SKR1 is unlikely a receptor for a ligand within the subgroup. The similarity of the cell and tissue expression pattern of SKR1 to TGFβRII further suggests that the ligand for SKR1 is likely to be within the TGF-β1–5 branch of the superfamily (5). However, the BMP-5, -6, and -7 subgroup which is expressed significantly in non-skeletal tissues as lung, liver, and kidney (22, 23) cannot be eliminated. Identification and characterization of the ligand for SKR1 is essential to understanding its function in the large number of tissues and cells in which it is expressed.

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4. Mathews, L. S., Vale, W. W., and Kinzler, K. R. (1992) Science 255, 1702-1705
5. Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A., and Ladash, H. F. (1992) Cell 68, 775-785
6. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42-52
7. Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., and Sowadski, J. M. (1992) Ann. Rev. Cell Biol. 8, 429-462
8. Georgi, L. L., Albert, P. S., and Riddle, D. L. (1990) Cell 61, 635-646
9. Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., and Sowadski, J. M. (1992) Cell 68, 775-785
10. Mansson, P. E., Adams, P., Kan, M., and McKeehan, W. L. (1989) Cancer Res. 49, 2485-2494
11. Hou, J., Kan, M., McKeehan, K., McBride, G., Adams, P., and McKeehan, W. L. (1991) Science 251, 665-668
12. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5892-5896
13. Xu, J., Nakahara, M., Crabbb, J. W., Shi, E., Matuo, Y., Fraser, M., Kan, M., Hou, J., and McKeehan, W. L. (1992) J. Biol. Chem. 267, 17792-17801
14. Krummen, L. A., Woodruff, T. R., DeGuzman, G., Cox, E. T., Baly, D. L., Mann, E., Garg, S., Wong, W. L., Costum, P., and Mather, J. P. (1993) Endocrinology 132, 431-443
15. Lucas, C., Pembly, R. M., Maki, V. R., Wong, W. L., and Palladino, M. A. (1991) Methods Enzymol. 188, 303-316
16. Patalkar, V. M., Hammond, R. G., and Reddi, A. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3097-3101
17. Hou, J., Kan, M., Wang Y., Xu, J., Nakahara, M., McBride, G., McKeehan, W., and McKeehan, W. L. (1990) J. Biol. Chem. 267, 17304-17308
18. Hoshi, H., Kan, M., Chen, J., and McKeehan, W. L. (1989) In Vitro Cell & Dev. Biol. 25, 309-320
19. Myoken, Y., Kan, M., Sato, C. H., McKeehan, W. L., and Sato, J. D. (1990) Exp. Cell Res. 191, 299-304
20. Kan, M., Huang, J., Mansson, P. E., Yamasita, H., Carr, R., and McKeehan, W. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7432-7436
21. Kan, M., Yan, G., Xu, J., Nakahara, M., and Hou, J. (1990) In Vitro Cell & Dev. Biol. 28A, 515-520
22. Lyons, K., Greygar, J. L., Lee, A., Hashmi, S., Lindquist, P. H., Chen, E. Y., Hogan, B. L. M., and Dennis, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4554-4558
23. Kingsley, D. M., Bland, A. E., Gruber, J. M., Marker, P. C., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (1982) Cell 71, 399-410