graphite rod, the soot contained ~10.1% C_{60}. For residue rods with buckytubes, the percentage yield of C_{60} was in the range of 20 to 60%, depending on the percentage of buckytubes present in the rod. The relative amount of higher fullerene (greater than C_{70}) has also increased from our soot separation. The fullerene yield correlated well with the number density of buckytubes in the residue rod, that is, the larger the number of buckytubes, the more the yield of fullerenes.

The possible steps in closed shell fullerene formation are outlined in Fig. 4. The first step includes tearing of graphite sheets in the vicinity of the arc. The folding of the graphite sheets would lead to buckytube formation. Recent theoretical work (*11, 14*) suggests that a tubular morphology is favored over a flat sheet under certain geometrical conditions. The number of sheets and their length determine the diameter of the buckytubes and their linear length. These buckytubes may become fragmented at the arc tip, which would lead to further closing of the open end or ends of the fragmented buckytubes in the carbon mist of the arc. Statistical consideration may dictate the critical number of carbon atoms and their configuration (that is, number of hexagons and pentagons). Those that obey the critical conditions may close their shells on separation or, alternatively, as they pass through the stream of atomic carbon in the arc, escaping as fullerenes in either case. Those that do not satisfy the number and geometrical requirements may rapidly dissociate to form lower fullerenes or carbon residue or both. The higher yield of C_{60} fullerenes may be associated with the growth kinetics of buckytubes where the innermost shell is perhaps all alone in the vicinity of the arc. The fragmentation of the inner shells is likely to produce more C_{60} than higher fullerenes. The closed shell fullerenes, thus formed, may either dissociate to lower fullerenes or residue or both if the geometry and number criteria are not satisfied or may land as fullerenes (C_{60}, C_{70}, and so forth).

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Betacellulin: A Mitogen from Pancreatic β Cell Tumors

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Betacellulin, a member of the epidermal growth factor family, has been identified in the conditioned medium of cell lines derived from mouse pancreatic β cell tumors. Betacellulin is a 32-kilodalton glycoprotein that appears to be processed from a larger transmembrane precursor by proteolytic cleavage. The carboxyl-terminal domain of betacellulin has 50 percent sequence similarity with that of rat transforming growth factor α. Betacellulin is a potent mitogen for retinal pigment epithelial cells and vascular smooth muscle cells.

It is increasingly evident that carcinogenesis involves a series of genetic and epigenetic changes that together produce a malignant phenotype. Many of these changes are implicated in the aberrant growth control of the tumor cells themselves (*1, 2*) and in the induction of new blood vessel growth (angiogenesis) to the tumors (*3*). Two transgenic mouse models of tumorigenesis have provided evidence that angiogenesis is activated in the late preneoplastic stages, which suggests that the switch to the angiogenic phenotype is a discrete event (*4, 5*). One experimental avenue to study the mechanisms that control both tumor cells and the vascular system is to identify molecules secreted by tumor cells that have mitogenic activity on one or another of the cell types involved in tumor growth. During the course of such experiments, we have identified a growth factor and refer to it as betacellulin.

RIP1-Tag2 mice carry a hybrid oncogene composed of a rat insulin gene regulatory region fused to the coding region of the simian virus 40 (SV40) T antigen gene. These mice develop pancreatic β cell carcinomas (insulinomas) in a multistep process (*6*), and a series of cell lines (called BTC) have been derived from these tumors (*7*). One cell line, BTC-3, is being used for a systematic study of tumor growth and angiogenesis factors. Betacellulin was initially detected as a mitogen for Balb/c 3T3 cells in the conditioned medium of this and another insulinoma-derived cell line, BTC-JC10, and was purified to apparent homogeneity (*8*). Approximately 6 μg of pure betacellulin was isolated from 200 liters of medium. The overall purification was 170,000-fold, with 4% recovery. Purified betacellulin migrated as a single band in SDS–polyacrylamide gel electrophoresis, with an apparent molecular mass of ~32 kD (Fig. 1A). We recovered the mitogenic activity of betacellulin on Balb/c 3T3 cells by extracting the 32-kD protein band from the polyacrylamide gel. Betacellulin stimulated the proliferation of retinal pigment epithelial and vascular smooth muscle cells at a concentration of ~30 pM (1 ng/ml) but did not stimulate the growth of several other cell types, such as endothelial cells and fetal lung fibroblasts (Fig. 1B).

Microsequencing of purified betacellulin protein with a protein sequencer (ABI 477; Applied Biosystems, Foster City, California) provided a partial NH_2-terminal amino acid sequence and three internal sequences. The partial NH_2-terminal sequence A was identified as Asp-Gly-(X)-Thr-(X)-Arg-Thr-Pro-Glu-Thr-Asn-Gly-Ser-Leu-(X)-Gly-Ala-Pro-Gly-Glu-Glu-Arg-Thr (where X represents unidentifiable residues). A computer search through the translated GenBank and NBRE (National Biomedical

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Research Foundation) protein database disclosed no similar peptides. The three internal sequences of betacellulin (B, C, D) were determined after fragmentation of the purified protein with endoproteinase Lys-C. Sequence B was identified as Thr-His-Phe-Ser-Arg-Cys-Pro-Lys, sequence C as His-Tyr-Cys-Ile-His-Gly-Arg-Cys-Arg-Phe-Val-Val-Asp-Glu-Gln-Thr-Pro-Ser-Cys-Ile-Cys-Glu-Lys, and sequence D as Gly-Tyr-Phe-Gly-Ala-Arg-Cys-Glu-Arg-Val-Asp-Leu-Phe-Tyr. These three sequences exhibited substantial homology with both rat and human transforming growth factor α (TGF-α) (*9, 10*). On the basis of the amino acid sequence data, we cloned cDNA encoding mouse betacellulin (*11*) and determined its nucleotide sequence (Fig. 2).

The sequence data revealed that the mature form of betacellulin consists of 80 amino acids and is derived from a larger precursor protein. If the methionine located 31 amino acids upstream of the mature

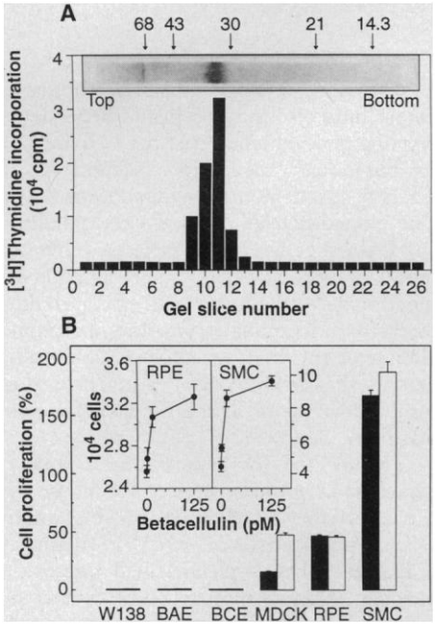

Fig. 1. Biological activities of betacellulin. (**A**) Purified betacellulin (~200 ng per lane) was subjected to 15% SDS–polyacrylamide gel electrophoresis in the absence and presence of β-mercaptoethanol. In both cases, a single band with a molecular mass of ~32 kD was visualized by silver stain. To recover the biological activity of betacellulin from the gel, we silver-stained one lane of a nonreducing slab gel (inset), and the other lanes were each cut into 26 slices. Molecular size standards are shown at the top in kilodaltons. The top and bottom of the gel are indicated. The gel slices were extracted individually with saline, and we tested growth factor activity by measuring [^{3}H]thymidine incorporation into the DNA of quiescent monolayers of Balb/c 3T3 cells (*27*). (**B**) The mitogenic activity of betacellulin was also tested on bovine aortic endothelial cells (BAE), bovine capillary endothelial cells (BCE), human fetal lung fibroblasts (WI38), canine kidney epithelial cells (MDCK), retinal pigment epithelial cells (RPE), and vascular smooth muscle cells (SMC). Cell proliferation was assayed as percent over negative control in 24-well plates (10,000 cells and 0.5 ml of medium per well). After ~72 hours, cells were detached with 0.05% trypsin and counted in a Coulter counter. Filled boxes indicate 100 pM betacellulin; open boxes indicate 100 pM EGF. Data given are means ± SEM; n = 3.

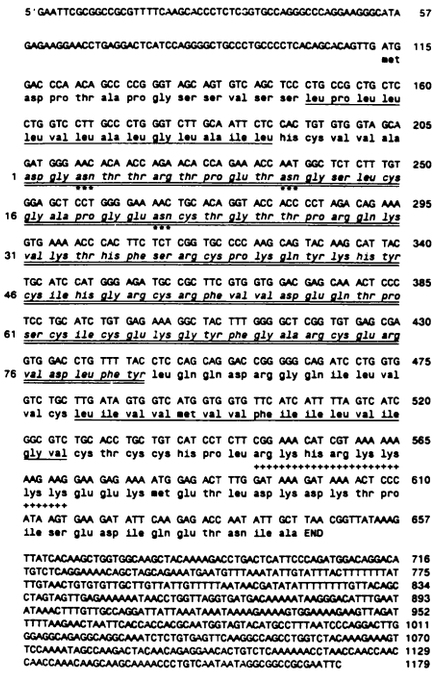

Fig. 2. Nucleotide sequence of betacellulin cDNA (numbered on the right) and deduced amino acid sequence. The nucleotide sequence has been deposited in GenBank (L08394). Betacellulin comprises 80 amino acids (double underlined and numbered on the left) and appears to be highly glycosylated, as suggested by the presence of three potential sites of N-glycosylation (asterisks) and by the fact that purified betacellulin produces a diffuse band with a molecular mass of ~32 kD on SDS-polyacrylamide gels. The amino acid sequence of purified betacellulin predicts a protein of 9 kD, as does the cDNA sequence in comparison to mature TGF-α. However, when a portion of the cDNA encompassing only the 80–amino acid mature form of betacellulin was expressed in *Escherichia coli*, an active protein of 18 kD was produced. Moreover, isolation of betacellulin from COS-7 cells transfected with full-length betacellulin cDNA revealed a protein of ~32 kD, which after digestion with *N*-glycanase produced two proteins of 24 and 18 kD (*22*). These results suggest that betacellulin expressed in both bacteria and mammalian cells exists as a firmly linked 18-kD dimer that is resistant to denaturation by SDS and sulfhydryl reducing agents. The predicted amino acid sequence of betacellulin shows that Asp^1 through Thr^{23} coincides with sequence A with the exception of Asn^{21} and Cys^{22}, which presumably resulted from erroneous amino acid identification. Thr^{33} through Lys^{40}, His^{44} through Lys^{66}, and Gly^{67} through Tyr^{80} coincide with sequences B, C, and D, respectively. The COOH-terminal domain of mature betacellulin, Val^{31} through Tyr^{80}, has 50% homology with TGF-α. Two hydrophobic domains are indicated by single underlines, and the basic sequence is marked by pluses.

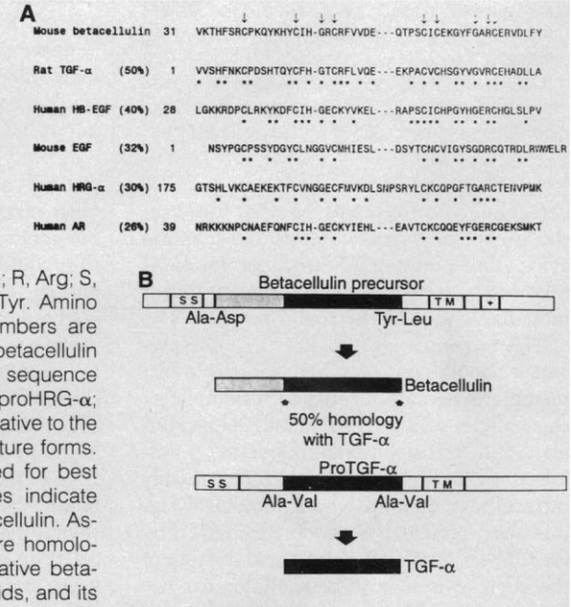

Fig. 3. Structural relation of mouse betacellulin and other members of the EGF family. (**A**) Alignment of the COOH-terminal 50–amino acid sequence of betacellulin with the corresponding sequences of other members of the EGF family. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Amino acids strictly conserved by all members are indicated by arrows on top of the betacellulin sequence. The heregulin (HRG-α) sequence begins with amino acid 175 of the proHRG-α; other polypeptides are numbered relative to the NH_2-terminal amino acids of the mature forms. Gaps in the sequences are inserted for best alignment. Numbers in parentheses indicate percent homology with mouse betacellulin. Asterisks indicate amino acids that are homologous with betacellulin. (**B**) The putative betacellulin percursor has 177 amino acids, and its mature form has 80 amino acids. ProTGF-α has 159 amino acids, and the mature protein has 50 amino acids. Similar to proTGF-α, the betacellulin precursor contains a signal sequence (SS) and a transmembrane domain (TM). The betacellulin precursor also appears to have a unique basic sequence (+).

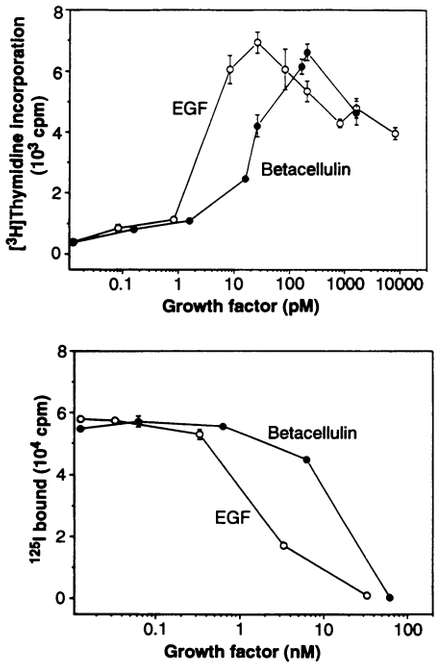

Fig. 4. Characterization of recombinant betacellulin. (**A**) Relative stimulation of [^{3}H]thymidine incorporation into the DNA of Balb/c 3T3 cells by mouse recombinant betacellulin and human recombinant EGF (R and D Systems). (**B**) Relative binding of mouse recombinant betacellulin and human recombinant EGF to human cells expressing the EGF receptor. The competitive ^{125}I-labeled EGF binding assay was performed as in (*29*). The ^{125}I-labeled EGF (85,000 cpm) (Amersham) was added to each well of a 24-well plate containing confluent MDA-MB 468 cells (American Type Culture Collection HTB 132) together with increasing amounts of betacellulin or EGF. The cells were incubated at room temperature for 1 hour and subsequently rinsed twice with cold DMEM containing bovine serum albumin (0.1%). To solubilize the cells, we added 0.3 N NaOH (0.2 ml) to each well, and the radioactivity was measured with a gamma spectrometer. Data given are means ± SD of two experiments.

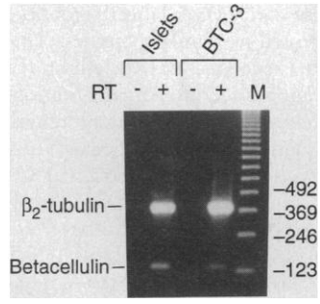

Fig. 5. Betacellulin mRNA expression. Analysis by RNA-PCR. Total RNA was extracted from BTC-3 cells and the islets of Langerhans isolated from a 10-week-old mouse. The RNA was reverse-transcribed into single-stranded cDNA by random hexamer priming in the absence or presence of reverse transcriptase (RT) (*30*). The cDNAs corresponding to betacellulin and to β_2-tubulin (used as an internal control) were amplified by PCR with specific primers for betacellulin (5′-CCTCACAGCACAGTTGATGG-3′ and 5′-GGTGTTCTGGTTGTGTTCCC-3′) and β_2-tubulin (5′-CAACGTCAAGACGGCCGTGTG-3′ and 5′-GACAGAGGCAAACTGAGCACC-3′) according to standard procedures (*31*). The PCR products were separated by electrophoresis on a 2% low-melting agarose gel and visualized by staining with ethidium bromide. The specific PCR fragments corresponding to betacellulin (132 bp) and β_2-tubulin (396 bp) are indicated on the left. Size markers (lane M) are indicated in base pairs on the right.

betacellulin corresponds to the initiator, the precursor would comprise 177 amino acids. This putative initiator methionine is followed by a hydrophobic sequence that is most likely part of the signal peptide. The cDNA sequence also predicts that the precursor should contain a hydrophobic transmembrane domain located 12 residues after the COOH-terminus of mature betacellulin. This transmembrane domain is followed by a highly basic and presumably intracellular domain whose biological significance remains to be determined. The COOH-terminal, 50–amino acid domain of the 80-residue mature betacellulin has the following homologies with the corresponding sequences of members of the epidermal growth factor (EGF) family: TGF-α, 50% (9); heparin-binding EGF (HB-EGF), 40% (*12*); mouse EGF, 32% (*13*); heregulin/neu differentiation factor (NDF), 30% (*14*, *15*); and amphiregulin (AR), 26% (*16*). The COOH-terminal domain also contains six conserved cysteine residues that are shared by all of these and other members of the EGF family (Fig. 3A).

We compared the organization of the putative precursor and the mature forms of betacellulin to the precursor (proTGF-α) and mature forms of TGF-α (Fig. 3B). The cleavage sites in proTGF-α that generate the NH_2-terminus and COOH-terminus of mature TGF-α are both located between alanine and valine (*9*, *17*). These neutral amino acids constitute the cleavage site for an elastase-like enzyme (*18*). It was demonstrated that enzymatic cleavage of membrane-anchored proTGF-α could be activated in response to serum factors and tumor-promoting phorbol esters and thus may be a regulatory step in the generation of soluble TGF-α (*19*). In contrast, cleavage of the mature betacellulin from its putative precursor appears to occur at sites with different amino acids (alanine–aspartic acid and tyrosine-leucine for the NH_2-terminal and COOH-terminal cleavages, respectively). This observation suggests that distinct and perhaps unknown proteases may release soluble betacellulin from a membrane-anchored form. Such a transition could be important in controlling the biological activity of betacellulin.

To demonstrate that the cloned cDNA sequence codes for functional betacellulin, we transfected betacellulin cDNA in a mammalian expression vector, pTB701 (*20*), into COS-7 cells and purified betacellulin from conditioned medium of the transfected cells (*21*). The purified recombinant betacellulin stimulated ^{3}H-labeled thymidine incorporation into Balb/c 3T3 cells in a dose-dependent manner (Fig. 4A), and it competed for the binding of ^{125}I-labeled EGF to the cell surface receptor of a human breast tumor cell line, MDA-MB 468, albeit with approximately one-tenth of the affinity that purified recombinant EGF had (Fig. 4B). Recombinant betacellulin also induced ligand-dependent tyrosine phosphorylation of the EGF receptor but not the neu/HER2 receptor (*22*). These results demonstrate the following. (i) The cloned cDNA codes for betacellulin. (ii) COS-7 cells are also able to generate proteases for the cleavage and production of functional betacellulin. (iii) Betacellulin binds to and stimulates tyrosine phosphorylation of the EGF receptor. COS-7 cells express the same SV40 T antigen as the insulinomas do in which betacellulin was originally identified.

The mRNA for betacellulin is widely expressed in normal tissues of the mouse. It was detected by Northern (RNA) blot analysis of polyadenylated RNA in thymus, lung, heart, liver, spleen, small intestine, pancreas, kidney, muscle, testis, and uterus, with substantially higher amounts found in lung, uterus, and kidney (*22*). Several mRNA species (4.2, 3.0, 1.2, and 0.9 kb) were detected. Betacellulin mRNA was also detected in mouse sarcoma 180 and fibrosarcoma BPV-11 cell lines. Moreover, a human homolog of betacellulin cDNA has been isolated from a breast adenocarcinoma cell line MCF-7 (*23*). In contrast, betacellulin mRNA was present in small amounts or was undetectable in several other tumor cell lines, including B-16 melanoma, Lewis lung carcinoma, Meth A sarcoma, colon carcinoma–26, and glucagonoma αTC1 (*22*). Betacellulin mRNA was also detected in pancreatic islets from normal (nontransgenic) mice and in BTC-3 cells (Fig. 5) by RNA polymerase chain reaction (PCR) analysis (*24*), albeit in somewhat smaller amounts than in lung, uterus, and kidney (*22*).

There are a number of implications arising from the structure and mitogenic specificity of betacellulin. Our finding that this growth factor is expressed in insulinomas and certain other tumor cell lines suggests that it may contribute to the phenotype of these cancers. The structural characteristics of betacellulin indicate that it is produced

from a precursor molecule by proteolytic cleavage, which raises the possibility of tissue-specific regulation through protease activation. The structural homology of betacellulin with members of the EGF family and the relatively low affinity of betacellulin for the classical EGF receptor suggests that betacellulin might act through another receptor. The possibility exists that betacellulin is primarily a ligand for another known member of the EGF receptor family, which includes the *erb*B2 (*neu/HER2*) (*25*) and *erb*B3 oncogene products (*26*). However, our analyses of ligand-dependent tyrosine phosphorylation indicate that the *erb*B2 receptor is not stimulated by betacellulin (*22*). Thus, *erb*B3 or another receptor remain as candidates for the primary betacellulin receptor. Finally, the fact that betacellulin is mitogenic for smooth muscle and retinal pigment epithelial cells and is produced by proliferating pancreatic β cells suggests the possibility that release of betacellulin from β cells could have a role in the vascular complications associated with diabetes.

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8. BTC-3 cells were grown in 900-cm^2 roller bottles containing 125 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum. After 4 days, the medium was replaced with serum-free medium. The serum-free medium was harvested 48 to 72 hours later and replaced with DMEM containing 5% calf serum. A variant insulinoma-derived cell line, BTC-JC10, was also used as a source of betacellulin protein and mRNA. Identical NH_2-terminal sequence information was obtained from material purified from the two cell lines. To purify betacellulin, we applied the concentrated medium to a BioRex 70 (Bio-Rad) column equilibrated with 10 mM tris, pH 7, at 4°C. The mitogenic activity eluted at ~0.3 M NaCl when the column was washed with a gradient of 0 to 0.6 M NaCl. The active fractions were brought to 1.5 M $(NH_4)_2SO_4$ and applied to a phenyl-Sepharose (Pharmacia) column equilibrated at 1.5 M $(NH_4)_2SO_4$ and 10 mM potassium phosphate buffer, pH 7, at 4°C. The activity eluted at ~1.2 M $(NH_4)_2SO_4$ when the column was washed with a gradient of 1.5 to 0 M $(NH_4)_2SO_4$. The active fractions from the phenyl-Sepharose column were subjected to fast protein liquid chromatography (FPLC) with a heparin-affinity column equilibrated with 10 mM tris at pH 7. The activity eluted at ~0.3 M NaCl when the column was washed with a gradient of 0 to 0.6 M NaCl. Finally, the active fractions were subjected to high-performance liquid chromatography (HPLC) on a C_4 reversed-phase column equilibrated with 10% acetonitrile in 0.1% trifluoroacetic acid. The activity eluted at ~20% acetonitrile when the column was washed with a 10 to 35% gradient of acetonitrile. We repeated the HPLC step once to maximize purity. The purification was guided by an assay that measures the stimulation of [^{3}H]thymidine incorporation into the DNA of quiescent monolayers of Balb/c 3T3 cells (*27*).
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21. We constructed an expression plasmid for betacellulin cDNA, pTB1491, by inserting a 1.2-kb cDNA from pTB1489 into an Eco RI site of pTB701 (*20*). COS-7 cells (3 × 10^5 cells per 60-mm dish) were transfected with 10 μg of plasmid DNA by the calcium phosphate co-precipitation method [F. L. Graham and A. J. van der Eb, *Virology* **54**, 536 (1973)]. The next day the culture medium was changed to DMEM supplemented with 0.5% fetal calf serum (5 ml), and the cells were incubated for 2 days. The medium was collected, and we used it to prepare betacellulin. Approximately 6 μg of recombinant betacellulin was purified from 2 liters of culture medium.
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SH2-Containing Phosphotyrosine Phosphatase as a Target of Protein-Tyrosine Kinases

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A mouse phosphotyrosine phosphatase containing two Src homology 2 (SH2) domains, Syp, was identified. Syp bound to autophosphorylated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors through its SH2 domains and was rapidly phosphorylated on tyrosine in PDGF- and EGF-stimulated cells. Furthermore, Syp was constitutively phosphorylated on tyrosine in cells transformed by v-*src*. This mammalian phosphatase is most closely related, especially in its SH2 domains, to the *corkscrew* (*csw*) gene product of *Drosophila*, which is required for signal transduction downstream of the Torso receptor tyrosine kinase. The *Syp* gene is widely expressed throughout embryonic mouse development and in adult tissues. Thus, Syp may function in mammalian embryonic development and as a common target of both receptor and nonreceptor tyrosine kinases.

Protein tyrosine phosphorylation is a principal biochemical mechanism by which hormones and growth factors regulate cell proliferation, differentiation, and metabolism. Activated receptor protein-tyrosine kinases (PTKs) apparently stimulate intracellular signaling pathways by binding and phosphorylating regulatory cytoplasmic proteins (*1–5*). These cytoplasmic targets share a common structural element, SH2 domain, that mediates their association with specific phosphotyrosine-containing sites on autophosphorylated receptors and thereby controls the initial interactions of receptors with their substrates (*6*). Because cell growth and development are tightly controlled physiological processes, dephos-

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