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Nucleotide sequence of a cDNA coding for rat *hck* tyrosine kinase and characterization of its gene product

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Abstract. The *hck* gene is member of *src* family of non-receptor type tyrosine kinases. Here we report the nucleotide sequence of the rat *hck* eDNA of 1·94 kb. The nuclcotide sequence shows an open reading frame coding for a polypeptide of 503 amino acids. A vector expressing a fusion protein of glutathione-S-transferase with 82 amino acids of the N-terminal region of *hck* (from amino acids 32 to 113) was constructed, Using this bacterially expressed fusion protein antibodies were prepared which recognize the cellular *hck* gene product. These antibodies identified, by immunoblotting, two polypeptides of 56 and 59 kDa in rat spleen where *hck* transcripts are present at high level. Immunoprecipitated *hck* polypeptides were enzymatically active and were autophosphorylated in the presence of ATP and Mg^{2+}. 1mmunoprecipitated *hck* could phosphorylate exogenous substrates. Treatment of immunoprecipitated *hck* by a purified protein tyrosine phosphatase decreased its enzymatic acitivity. Our results suggest that the enzymatic activity of *hck* tyrosine kinase is regulated by phosphorylation and dephosphorylation.

Keywords. *hck;* protein tyrosine kinase; enzyme regulation; nucleotide sequence; auto-phosphorylation.

1. Introduction

Protein tyrosine kinases have been implicated to be key regulatory molecules in the signal transduction pathways controlling many vital cellular processes. These protein tyrosine kinases have been broadly classified into two classes (Swarup et al 1984; Hunter and Cooper 1985). The transmembrane receptor tyrosine kinases have direct involvement in signal transduction pathways (Ulrich and Schlessinger 1990). The non-receptor kinases are intracellular proteins which lack both trans-membrane and extracellular domains, but are capable of membrane association (Hunter and Cooper 1985; Sreevastava 1990; Bolen 1993). The members of the *src* gene family *src, lck, lyn, fyn, yes, fgr* and *blk* share common structural features and many of them are expressed in various hematopoietic cells (Perlmutter et al 1988; Eiseman and Bolen 1990). Recent studies have shown that *fyn, lyn* and *lck* are associated with cytoplasmic domains of different cell surface receptors (Rudd *et al* 1988; Veillette *et al* 1988; Samelson et al 1990; Yamanashi *et al* 1991), suggesting, therefore, that the members of *src* gene family might play important roles in signal transduction. Strong evidence in favour of this comes from the studies demonstrating direct physical and functional association of *fyn* tyrosine kinase with the antigen receptors of T cells (Samelson et al 1990).

We have earlier isolated cDNA clones coding for *hck* tyrosine kinase from a rat spleen cDNA library (Rema and Swarup 1990). Here we report the complete nucleotide sequence of rat *hck* cDNA. The polypeptide products of the rat *hck* gene were identified by preparing antibodies using a fusion protein of *hck* with glutathione-S-transferase (GST), made in *Escherichia coli,* as antigen. Using these antibodies we have characterized the enzymatic properties of immunoprecipitated *hck* protein; our results suggest that its kinase activity is regulated by phosphorylation and dephosphorylation.

2. Materials and methods

2.1 *Analysis of RNA*

Total RNA was isolated from various tissues using the method of Chomczynski and Sacchi (1987). mRNA was purified by oligo dT cellulose (Pharmacia) column chromatography. Total RNA (10 µg) or mRNA (5·µg) was separated on 1% agarose gel containing 2·2 M formaldehyde and transferred to nitrocellulose membrane. To ensure that equal amounts of RNA were present, 0·5 µg of ethidium bromide was added to the (25 µl) samples prior to loading, to visualize RNA after the gel was run. Hybridization was done at 50°C as described previously (Swarup *et al* 1991). The blots were washed with 0·2 × SSPE + 0·1% SDS at 60°C and kept for autoradiography at - 70°C. The *hck* probe was labelled by nick translation.

2.2 *Isolation of rat hck cDNA clones and sequence analysis*

Rat *hck* cDNA clones were isolated from a rat spleen cDNA library (Rema and Swarup 1990). Complete nucleotide sequence of the largest clone Ll15 (which contains 1940 nucleotides) was determined by sequencing both strands of DNA using dideoxy chain termination method (Sanger *et al* 1977) as modified for double stranded DNA by Chen and Seeburg (1985). Smaller clones of *hck* and various restriction fragments of the large clone L115 were subcloned into pUC 19 for sequence analysis. This nucleotide sequence has been deposited with EMBL data bank (accession number X 62345). Standard molecular cloning experiments such as ligations, transformation etc., were carried out as described by Sambrook *et al* (1989).

2.3 *Preparation of tissue samples*

Soluble and particulate fractions from various tissues were prepared as described previously (Swarup and Subrahmanyam 1989). Normal macrophages were collected from the rat by peritoneal lavage, by injecting l0 ml PBS. The cells were then washed with PBS and used for further experiments.

2.4 *Preparation of hck antibodies*

A 246 bp *Bam*HI-*Hinc*II fragment (amino acids 32 to l13) from the 5′ coding region of the rat *hck* cDNA was ligated with *Bam*HI and *Sma*I digested pGEX-1 expression vector (Smith and Johnson 1988). The resulting construct, pGEX-*hck*, expressed a fusion protein with GST carrying *hck* sequences at the C-terminal end. This fusion protein band of 35 kDa was electroeluted from polyacrylamide gels and used to inject several Balb/c mice with complete Freund's adjuvant. Two further injections were given with Freund's incomplete adjuvant at 15 day intervals and blood was collected 4 days after the 3rd injection. Antibody titres were monitored by immunodiffusion and immunoblotting.

2.5 *Production of hck monoclonal antibodies*

Splenocytes were collected from a mouse showing high *hck* antibody titre after boosting with the bacterially expressed GST-*hck* fusion protein intraperitoneally. These splenocytes were fused with Sp2/0 myeloma cells by using polyethylene glycol method. Hybrid cells were dispersed in HAT-IMDa over a layer of mouse peritoneal macrophage feeder cells (Fazekas de St. Groth and Scheidegger 1980). Supernatants from HAT resistant colonies were checked for antibody secretion by enzyme linked immuno filtration assay (ELIFA) and Western blotting and the positive clones propagated. Colonies giving a good positive reaction were cloned by limiting dilution. The hybridoma cell lines were maintained in IMDM supplemented with 10% FCS and antibiotics.

2.6 *Immunoblotting*

Proteins fractionated by SDS-PAGE were transferred electrophoretically to Immobilon-P membranes. The membranes were blocked using 1% horse serum in TBST-gelatin (10 mM Tris-CI, pH 8·0, 150 ma NaCl, 0·05% Tween E0 and 0·2% gelatin) for 1 h. The blots were incubated with either polyclonal antibody diluted 1 : 1000 or hybridoma supernatant diluted 1 : 10 for 2 h. After washing for 2 h, the blots were incubated with alkaline phosphatase conjugated anti mouse IgG for 1 h. After washing, immuno reactive bands were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

For competition experiments 10 µg of purified GST-*hck* fusion protein of 35 kDa was incubated at room temperature for 30 min with 5 µI of antiserum in a volume of 500 µI and then used as primary antibody after diluting to 5 ml in TBST.

2.7 *Immunoprecipitation, kinase assay and phosphates treatment*

Particulate fraction (0·l mg protein) of rat spleen was extracted with 0·5 ml of IP buffer (1 × IP buffer contains 150 mM NaCI, 1% Triton, 0·5% deoxycholate, 2 mM EDTA, E mM PMSF, E µg/ml soyabean trypsin inhibitor and 2 µg/ml leupeptin) on ice and centrifuged at 15,000 *g* for 20 min. Incubation with antibody or control

medium was carried out for 1 h at 4°C by gentle mixing. Preswollen protein A-sepharose beads were added and further mixed for 30 min. The beads were centrifuged and washed 5 times with IP buffer.

Phosphorylation of the immunoprecipitated *hck* was carried out in 25 mM Tris-CI, pH 7·5, 10 mM $MgC1_2$ 1 mM DTT, 2 mM PMSF, and protease inhibitors (leupeptin, antipain, soyabean trypsin inhibitor) at 2 μg/ml, 1 μM [γ-^{32}P]ATP (specific activity 5×10^4 cpm/pmol) in a total volume of 0·05 ml. After incubating at 30°C for 10 min the reaction was stopped by adding 0·05 ml of 2 × SDS sample buffer and boiled for 3 min. Exogenous substrate, 10 μg of poly (Glu^4, Tyr^1) was included wherever indicated. Treatment with phosphatase was carried out at 30°C for 10 min in the same buffer but without $MgC1_2$ or ATP. After phosphatase treatment the kinase assay was started by adding ATP, $MgC1_2$ and sodium orthovanadate (100 μM). The phosphatase used was a protein-tyrosine phosphatase expressed in *E. coil* (see below) and was added at 0·5 μg (2-3 μl) per assay.

2.8 *Purification of a protein tyrosine phosphatase made in E. coli*

A protein tyrosine phosphatase, PTP-S, has been expressed in *E. colic* at high level by constructing an expression vector (Radha *et al* 1993). This phosphatase was extracted from *E. coli* cells carrying the expression vector as described previously (Radha *et al* 1993). This extract was applied on a DEAE-Sephacel column, washed and the phosphatase was eluted with 0·1 M NaCl in the column buffer (25 mM Tris-C1, pH 7·l, 1 mM EDTA, 0·025% mercaptoethanol, 10% glycerol, 0·1% Triton X-100). Column fractions were assayed for tyrosine phosphatase activity using ^{32}P-labelled poly (Glu^4, Tyr^1) as substrate as described by Swarup and Subrahmanyam (1989). The purified enzyme was stored in 50% glycerol made in column buffer. This storage buffer was used as control buffer for phosphatase treatment experiments.

3. Results

3.1 *Nucleotide sequence of rat hck*

The rat *hck* cDNA clones were isolated by screening a spleen cDNA library (Rema and Swarup, 1990). The nucleotide sequence of the largest clone (1.94 kb) showed an open reading frame coding for a polypeptide of 503 amino acids beginning after 212 nucleotides at the 5′ end (figure 1). Figure 2 shows a comparison of first 80 amino acids of human, mouse and rat *hck* gene products starting from the initiator ATG codon, which is numbered + 1. Human and mouse *hck* sequences are taken from published papers (Quintrell *et al* 1987; Ziegler *et al* 1987; Holtzman *et al 1*987). Translation of mouse *hck* can also initiate from a CTG codon located 21 codons 5′ of the ATG codon (Lock *et al* 1991). This CTG codon, beginning at nucleotide 150 (figure l) is also conserved in rat *hck* sequence and is numbered –21 in figure 2. The rat and mouse *hck* proteins show 97% identity (16 substitutions) whereas rat and human *hck* and proteins show 89% identity (53 substitutions and 2 single amino acid deletions in rat *hck).* Most of the differences (11 out of 16) in amino acids between mouse and rat *hck* protein are located within the first 100 amino acids. This is also the case with human protein in which 29 out of 55

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 GA ATT CGG TCT CAG ACG GGT GGG AGG GAA CCA AAG TCG CCG GTA AAG GCG GCT CTG ACC   59
CGC TCG GAG CGC CAA CGC AGC CTC CGT AGC CCG CAA GTC TTC GTC GCT TGC TCC GGG CTC  119
TCG AGT CCG GGG CCA CCA GGG GCC GCG GCG CTG GGG GGT CGT TCG AGC TGC GAG GAT CCG  179
GGC TGC CCG CGA GGC GAA GGG CGG GTG CCC AGG ATG GGA TGT GTG AAG TCC AGG TTC CTC  239
                                          Met Gly Cys Val Lys Ser Arg Phe Leu

CGA GAA GGA AGC AAG GCC TCA AAA ATA GAG CCA AAT GCC AAC CAG AAA GGC CCT GTG TAT  299
Arg Glu Gly Ser Lys Ala Ser Lys Ile Glu Pro Asn Ala Asn Gln Lys Gly Pro Val Tyr

GTG CCG GAT CCC ACG TCC CCT AAG AAG CTG GGA CCG AAC AGC ATC AAC AGC CTG CCC CCG  359
Val Pro Asp Pro Thr Ser Pro Lys Lys Leu Gly Pro Asn Ser Ile Asn Ser Leu Pro Pro

GGG GTC GTG GAG GGC TCT GAG GAC ACC ATT GTG GTC GCA CTG TAC GAC TAT GAG GCC ATT  419
Gly Val Val Glu Gly Ser Glu Asp Thr Ile Val Val Ala Leu Tyr Asp Tyr Glu Ala Ile

CAC CGT GAA GAC CTC AGC TTC CAG AAG GGA GAC CAG ATG GTG GTT CTG GAG GAG TCT GGG  479
His Arg Glu Asp Leu Ser Phe Gln Lys Gly Asp Gln Met Val Val Leu Glu Glu Ser Gly

GAG TGG TGG AAG GCC CGT TCC CTG GCT ACC AAG AAA GAA GGC TAT ATC CCA AGC AAT TAT  539
Glu Trp Trp Lys Ala Arg Ser Leu Ala Thr Lys Lys Glu Gly Tyr Ile Pro Ser Asn Tyr

GTA GCT CGA GTT AAC TCT TTG GAG ACT GAG GAG TGG TTC TTC AAG GGT ATC AGC CGG AAG  599
Val Ala Arg Val Asn Ser Leu Glu Thr Glu Glu Trp Phe Phe Lys Gly Ile Ser Arg Lys

GAT GCA GAG CGC CAC CTG CTG GCT CCC GGG AAC ATG CTG GGC TCC TTC ATG ATC CGG GAC  659
Asp Ala Glu Arg His Leu Leu Ala Pro Gly Asn Met Leu Gly Ser Phe Met Ile Arg Asp

AGT GAG ACC ACC AAA GGG AGC TAC TCA CTT TCT GTT CGA GAC TTT GAC CCC CAG CAC GGA  719
Ser Glu Thr Thr Lys Gly Ser Tyr Ser Leu Ser Val Arg Asp Phe Asp Pro Gln His Gly

GAC ACG GTG AAG CAT TAT AAA ATC CGG ACA CTG GAC AGT GGA GGG TTC TAC ATC TCT CCG  779
Asp Thr Val Lys His Tyr Lys Ile Arg Thr Leu Asp Ser Gly Gly Phe Tyr Ile Ser Pro

AGG AGC ACC TTC AGC AGC CTG CAG GAA CTT GTC GTC CAC TAC AAG AGG GGG AAG GAT GGG  839
Arg Ser Thr Phe Ser Ser Leu Gln Glu Leu Val Val His Tyr Lys Arg Gly Lys Asp Gly

CTC TGC CAG AAG CTG TCA GTG CCC TGT GTG TCT CCC AAA CCC CAG AAG CCA TGG GAG AAA  899
Leu Cys Gln Lys Leu Ser Val Pro Cys Val Ser Pro Lys Pro Gln Lys Pro Trp Glu Lys

GAT GCC TGG GAG ATT CCT CGA GAA TCC CTC CAG ATG GAG AAG AAA CTG GGA GCC GGG CAG  959
Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Gln Met Glu Lys Lys Leu Gly Ala Gly Gln

TTT GGA GAA GTG TGG ATG GCC ACC TAC AAC AAG CAC ACC AAA GTG GCG GTG AAG ACA ATG 1019
Phe Gly Glu Val Trp Met Ala Thr Tyr Asn Lys His Thr Lys Val Ala Val Lys Thr Met

AAG CCA GGG AGC ATG TCT GTG GAG GCC TTC CTG GCA GAG GCC AAC CTG ATG AAG ACG TTA 1079
Lys Pro Gly Ser Met Ser Val Glu Ala Phe Leu Ala Glu Ala Asn Leu Met Lys Thr Leu

CAG CAT GAT AAA CTG GTG AAG CTG CAC GCC GTG GTC TCT CAG GAG CCC ACC TTT ATT GTC 1139
Gln His Asp Lys Leu Val Lys Leu His Ala Val Val Ser Gln Glu Pro Thr Phe Ile Val

ACC GAG TTC ATG GCC AAA GGA AGC CTG CTG GAC TTT CTC AAG AGT GAA GAA GGC AGC AAG 1199
Thr Glu Phe Met Ala Lys Gly Ser Leu Leu Asp Phe Leu Lys Ser Glu Glu Gly Ser Lys

CAG CCA CTG CCA AAA CTC ATT GAC TTC TCA GCC CAG ATT TCA GAG GGC ATG GCT TTC ATT 1259
Gln Pro Leu Pro Lys Leu Ile Asp Phe Ser Ala Gln Ile Ser Glu Gly Met Ala Phe Ile

GAG CAG AGG AAC TAC ATC CAC CGA GAC CTC CGG GCT GCC AAC ATC TTG GTT TCT GCA TCA 1319
Glu Gln Arg Asn Tyr Ile His Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Ser Ala Ser

CTG GTG TGT AAG ATC GCT GAC TTT GGA CTG GCA CGG ATC ATC GAG GAC AAT GAG TAC ACA 1379
Leu Val Cys Lys Ile Ala Asp Phe Gly Leu Ala Arg Ile Ile Glu Asp Asn Glu Tyr Thr

GCT CGG GAA GGA GCC AAG TTC CCC ATC AAG TGG ACA GCT CCT GAA GCC ATC AAC TTT GGC 1439
Ala Arg Glu Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu Ala Ile Asn Phe Gly

TCC TTC ACC ATC AAG TCA GAT GTC TGG TCC TTT GGT ATC CTG CTG ATG GAA ATC GTC ACC 1499
Ser Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Met Glu Ile Val Thr

TAC GGC CGG ATC CCT TAC CCA GGT ATG TCA AAC CCA GAG GTG ATT CGA GCA CTA GAG CAT 1559
Tyr Gly Arg Ile Pro Tyr Pro Gly Met Ser Asn Pro Glu Val Ile Arg Ala Leu Glu His

GGG TAC CGT ATG CCT CGA CCA GAT AAC TGC CCA GAG GAG CTC TAC AGT ATC ATG ATC CGC 1619
Gly Tyr Arg Met Pro Arg Pro Asp Asn Cys Pro Glu Glu Leu Tyr Ser Ile Met Ile Arg

TGC TGG AAG AAC CGT CCA GAG GAA CGG CCC ACT TTC GAA TAC ATC CAG AGC GTG CTG GAT 1679
Cys Trp Lys Asn Arg Pro Glu Glu Arg Pro Thr Phe Glu Tyr Ile Gln Ser Val Leu Asp

GAC TTC TAC ACG GCC ACT GAG AGC CAG TAT CAG CAG CAA CCT TGA TGG GCC GGA AGA ACA 1739
Asp Phe Tyr Thr Ala Thr Glu Ser Gln Tyr Gln Gln Gln Pro

TGA GCA CAG CCA GAA GCC CCA TCA GGG CCT TGA CAT GCT CGA CCT GCT GGG CCC ACT CTC 1799
AGA CGC CCC CTC CCC CAC ATT CCA GCT GTC GAG TGG AGG GAG AGG ACT TCA CAA TCT CTT 1859
TTT GAC TCT AGT CAT CTG CAA TCT GCC ATT CTC AGG GCC TCC AAG TTA GTG TTT CTC ATT 1919
TGC CTG GAA TGA ACT GAA TTC                                                   1940
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Figure 1 Nucleocide and deduced amino acid sequence of rat *lick* cDNA isolated from spleen cDNA library.

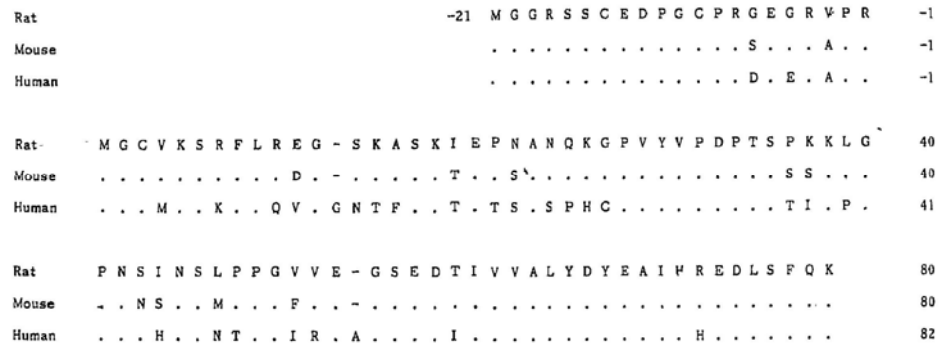

Figure 2 Comparison off amino terminal 100 amino acids off the rat, mouse and human *hck*. The initiator ATG codon is numbered as +1, Human and mouse sequences are taken from published papers (Quintrell et al 1987; Ziegler *el al* 1987; Holtzman *et al* 1987).

differences are located within the first 100 amino acids (figure 2). This suggests that the variability occurs mainly in the N-terminal region of the protein. There are three cysteine residues near the amino terminal end at positions –15, –10 and +3 which are conserved in rat, mouse and human (figure 2).

The rat *hck* protein shows a highly conserved tyrosine kinase domain beginning at amino acid 230. The tyrosine kinases of *src* family share conserved SH2 and SH3 domains *(src* homology domains 2 and 3, respectively). The SH2 (amino acids 109-229) and SH3 domains (amino acids 6l–l08) are also present in the rat *hck* in the N-terminal half of the protein. Amino acids 1-60 do not show any significant homology to any known protein and this region is therefore known as unique region. Two conserved Tyr residues, Tyr 388 and Tyr 499 are homologous to the autophosphorylation and negative regulatory phosphorylation sites, respectively, of the *src* kinase.

3.2 *Expression of hck transcripts*

We analysed the expression of *hck* transcripts in rat thymus, spleen and some other tissues, Northern hybridization was carried out with RNA from rat tissues, using a 550 base pair *Eco*RI-*Hinc*lI fragment from the 5′ end of the rat *hck* cDNA clone, L115. A major transcript of 2·2 kb was seen in the spleen. Another faint band of larger size was also observed. On longer exposure of the blot similar size transcripts were detected in thymus (figure 3), at very low level. *hck* transcripts could not be detected in brain and liver.

3.3 *Identification of hck gene product*

In order to identify the protein product coded by *hck,* antibodies were prepared using a fusion protein of GST and *hck* (amino acids 32 to 113) as immunogen. Construction of the vector pGEX-*hck* (figure 4) which expresses the 35 kDa GST-*hck* fusion protein in bacteria under the control of the inducible *tac* promoter is described in §E. The polyclonal antibodies raised in mice and the monoclonal

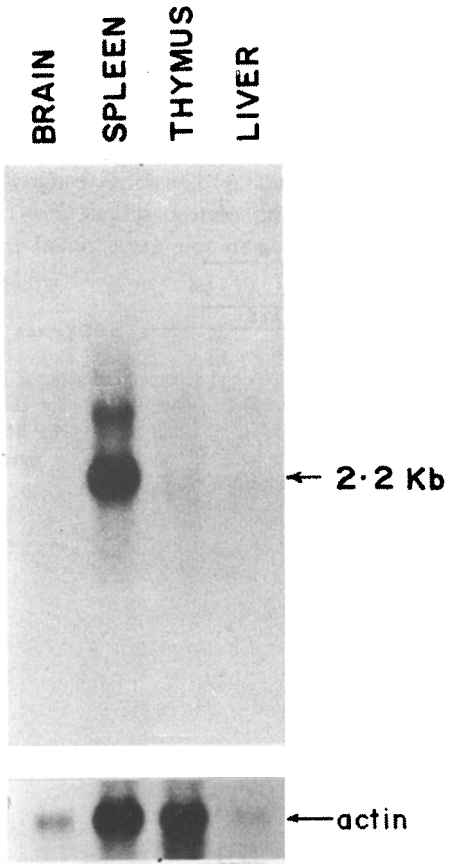

Figure 3. Analysis of *hck* transcripts from different rat tissues. Poly $(A)^+$ RNA (5 μg) from indicated tissues was hybridized to 5′ specific fragment of the rat *hck* cDNA as described in §2, The same blot was reprobed with actin (lower panel). The actin probe used was ß-actin which seems to be expressed al different levels in various tissues. However, an actin blot shows that the absence of *hck* transcripts in these tissues is not due to degradation of RNA.

antibody recognized the GST-hck fusion protein but not the GST itself by immunoblotting. These polyclonal as well as a monoclonal antibody identified two polypeptides of 59 and 56 kDa by immunoblotting in the particulate fraction of rat spleen (figure 5). When antibodies were preincubated with GST-*hck* fusion protein prior to immunoblotting, these two bands of 59 and 56 kDa were not observed suggesting that these polypeptides are not non-specifically precipitated.

3.4 *Kinase activity of hck protein*

Many protein tyrosine kinases can autophosphorylate upon incubation with ATP and divalent cations. The protein kinase activity of *hck* proteins was examined in the immunoprecipitate. For this purpose extracts were prepared from rat peritoneal macrophages or the particulate fraction of spleen and immunoprecipitation was

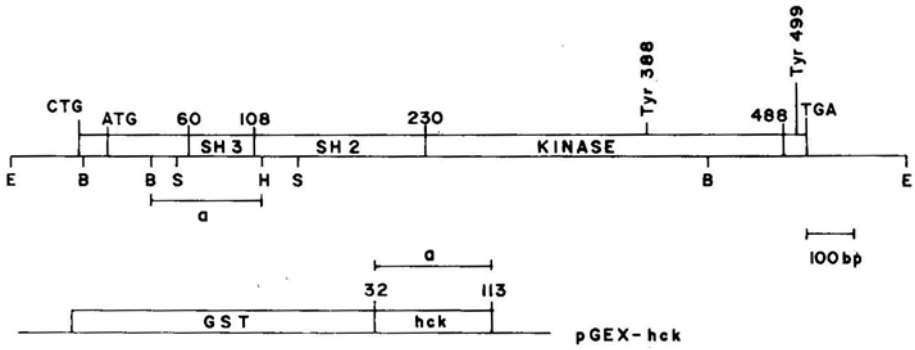

Figure 4. Restriction map of rat *hck* cDNA and schematic drawing of the vector *p*GEX-*hck* which expresses GST-*hck* fusion protein. The restriction sites are abbreviated. E, *Eco*RI; B, *Bam*HI (at nucleotides 173, 305, 1505); S, *Sma*I (358, 626); H, *Hinc*II (551). The numbers such as 32 and 113 are amino acids of rat *hck* as in figure 1. SH2 and SH3 are *sic* homology domains 2 and 3, respectively.

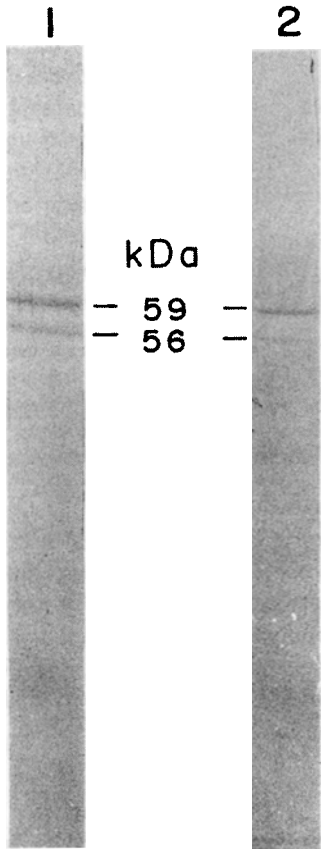

Figure 5. Identification of *hck* gene product by Western blotting. Particulate fraction from rat spleen (0·1 mg) was fractionated by SDS-PAGE on a 10% gel and analysed by Western blotting using polyclonal (lane I) or monoclonal (lane 2) *hck* antibody.

carried out using polyclonal antibodies and protein A-Sepharose as described under §2. The immunoprecipitate was incubated with [γ-^{32}P]ATP and Mg^{2+} and the resultant phosphorylated proteins were analysed by SDS-PAGE followed by autoradiography. Two polypeptides of 59 and 56 kDa were phosphorylated in the *hck* immunoprecipitate (figure 6, lanes 2, 3). This phosphorylation was alkali-stable (data not shown). The immunoprecipitated *hck* could phosphorylate poly (Glu^4, Tyr^1), a synthetic substrate for tyrosine kinases (figure 7, lane 1), but not BSA. When antibodies were preincubated with GST-*hck* fusion protein prior to immunoprecipitation, the 59 or 56 kDa phosphorylated bands were not observed (figure 6, lanes 1, 4).

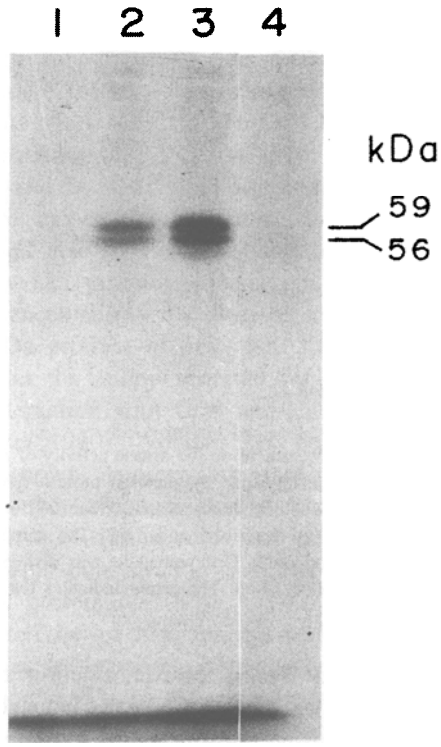

Figure 6. Protein kinase activity of *hck* gene product in the immunoprecipitate. Rat peritoneal macrophages (lanes 1, 2) or spleen particulate fraction (lanes 3, 4) were extracted and the extracts were subjected to immunoprecipitation and kinase assays as described in §2. The antibody was competed with GST-hck fusion protein (lanes 1, 4) for determining specificity. The samples were analysed by SDS-PAGE on a 12% gel followed by autoradiography.

3.5 *Regulation of kinase activity by phosphorylation*

Activity of some protein tyrosine kinases is regulated by autophosphorylation (Swarup and Radha, 1992). In order to test this possibility, immunoprecipitated *hck* was preincubated with ATP and Mg^{2+} followed by the addition of exogenous substrate; there was no increase in the tyrosine kinase activity of *hck.* This suggested

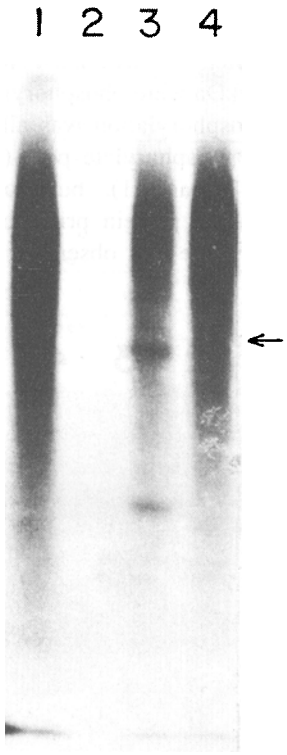

Figure 7. Effect of phosphatase treatment on the kinase activity of *hck,* Immunoprecipitated *hck* was incubated with control buffer (lane I), purified protein tyrosine phosphatase (lane 2) or phosphatase plus 100 μM sodium orthovanadate (lane 3) for [0 min at 30°C. The kinase reaction was then started as described under §2. The sample in lane 4 contained phosphatase during kinase reaction only. Orthovanadate was always present during kinose reaction so as to prevent dephosphorylation. The arrow indicates the position of phosphatase (M_r 47 kDa).

that either *hck* is not regulated by autophosphorylation or it is already in an activated, phosphorylated state. In case *hck* was already in an activated, phosphorylated state, dephosphorylation by a tyrosine phosphatase is expected to decrease its kinase activity. Therefore immunoprecipitated *hck* was treated with a purified protein-tyrosine phosphatase prior to kinase assay with poly (Glu^4, Tyr^1) as substrate; there was nearly complete loss of kinase activity of *hck* (figure 7, lane 2). This loss of kinase activity upon treatment with protein tyrosine phosphatase was prevented substantially if orthovanadate, a known inhibitor of this and other protein tyrosine phosphatases (Swarup *et al* 1982; Swarup and Subrahmanyam l989; Radha *et al* 1993) was included during phosphatase treatment (figure 7, lane 3). These observations suggest that phosphatase treatment inactivates *hck* by dephosphorylation. Therefore, the *hck* protein is in a phosphorylated, active state, which becomes less active or inactive by dephosphorylation. During incubation of *hck* with phosphatase in the presence of vanadate, the phosphatase was phosphorylated by *hck* (figure 7, lane 3). When phosphatase was added during the kinase assay, there was no effect on enzyme activity of *hck* (figure 7, lane 4) suggesting thereby that the phosphatase does not

interfere with the kinase activity in presence of vanadate. Because the PTP was added with vanadate, vanadate was always present during kinase assay. A labelled PTP band should be present in lane 4 but since poly (Glu, Tyr) is phosphorylated and gives a smear in that region the band cannot be seen. On shorter exposure one can see a PTP band but not as clearly as in lane 3. The phosphatase treated, inactive *hck* could be reactivated partially upon incubation with ATP (figure not shown). Incomplete reactivation may be due to phosphorylation of phosphatase by *hck* which may compete for autophosphorylation reaction.

4. Discussion

The nucleotide sequence of rat *hck* shows an open reading frame beginning with an ATG codon, coding for a polypeptide of 503 amino acids. In mouse *hck* translation can also initiate from an upstream CTG codon which was found to be conserved in the rat *hck* sequence also. This would give rise to a polypeptide of 524 amino acids. Identification of the *hck* gene product with antibodies showed two polypeptides of 59 and 56 kDa which are similar in size to mouse *hck* proteins. These polypeptides are enzymatically active, as shown by phosphorylation of exogeneous substrate and also by autophosphorylation.

A comparison of rat *hck* protein sequence in the N-terminal region with the mouse and human sequences shows that 3 cysteines are conserved at positions –15, –10 and +3. The 56 kDa product of *hck* will have only one conserved cysteine. Two cysteine residues in the amino terminal region of *lck* are known to play an important role in its interaction with CD4 and CD8 glycoproteins in T cells (Shaw *et al* 1989; Turner *et al* l990). Whether *hck* polypeptides interact with any cell surface proteins is not known. Another important point that emerges from sequence comparison (figure 2) is that although most of the differences are in the N-terminal 100 amino acids, the first l4 amino acids are identical in all the 3 species in the 59 kDa form of *hck*. This conservation suggests an important role for these 14 amino acids in the *hck* protein.

The protein kinase activity of *hck* appears to be regulated by phosphorylation. Treatment with phosphatase decreases kinase activity of *hck,* which suggests that in the immunoprecipitate, *hck* is in the phosphorylated, active state. The effect of phosphatase on kinase activity is due to dephosphorylation; this is suggested by the observation that the phosphatase inhibitor vanadate largely prevented inactivation of the kinase by phosphatase.

Regulation of protein kinase activity of low molecular weight tyrosine kinases by phosphorylation is known for a few kinases (Swarup and Radha 1992). We had previously shown that the kinase activity of TK-I, a tyrosine kinase isolated from |rat spleen, is regulated by autophosphorylation at tyrosine (Swarup and Subrahmanyam 1985a, b l989); dephosphorylation by a phosphatase inactivates this kinase (Swarup and Subrahmanyam 1989). This kinase could be isolated in the active state by including vanadate during purification; this preparation could not be activated further by incubation with ATP since it was already in the phosphorylated, active state (Swarup and Subrahmanyam l985, 1988, 1989). Another kinase isolated from bovine spleen was in the active state and could be inactivated by dephosphorylation (Kong *et al* 1988). The *hck* kinase activity seems to be regulated by phosphorylation in

the same manner as that of TK-I or the kinases from bovine spleen and lung (Kong *et al* 1988; Sreevastava and Chiasson 1988).

Regulation of protein kinase activity by phosphorylation is a very complex process which has been analysed in great detail in the case of *c-src* kinase (Hunter 1987). Phosphorylation at Tyr 416, which is also the *in vitro* autophosphorylation site, increases the kinase activity of *c-src* whereas phosphorylation at Tyr 527 inhibits its activity. When both the sites are phosphorylated the kinase activity is inhibited. Phosphorylation at Tyr 527, which is carried out by a tyrosine kinase named *csk*, inhibits kinase activity only when both SH2 and SH3 domains are intact. Deletion of SH2 or SH3 domains, or certain point mutations in these regions, affect inhibition of *src* kinase activity by phosphorylation at Tyr 527 (Superti-Furga *et al* 1993). Therefore, interaction of SH2 and SH3 domains with phosphorylated Tyr plays an important role in regulating kinase activity (Pawson and Gish 1992; Superti-Furga *et al* 1993). In addition, SH2 and SH3 domains are also involved in the interaction of these kinases with other proteins (Pawson and Gish 1992). The *hck* sequence shows conserved potential phosphorylation sites at Tyr 388 (homologous to Tyr 416 of *c-src*) and Tyr 499 (homologous to Tyr 527 of *c-src*). Which sites are phosphorylated in *hck in vitro* and *in vivo* are not known at present, Our results suggest that dephosphorylation of immunoprecipitated *hck* inhibits kinase activity, implying thereby that the *hck* protein is phosphorylated *in vivo* at a positive regulatory site.

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