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Polyoma middle tumor antigen interacts with SHC protein via the NPTY (Asn-Pro-Thr-Tyr) motif in middle tumor antigen

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ABSTRACT **Polyomavirus middle tumor antigen (MT) transforms a large number of cell types by binding to and modulating the activities of cellular proteins. Previous genetic analysis defined in MT an independent motif, NPTY (Asn-Pro-Thr-Tyr), required for transformation. This report demonstrates that NPTY is required for interaction between MT and SHC protein, a Src homology 2 (SH2)-containing protooncogene product implicated in activating Ras via association with GRB2 protein. SHC is phosphorylated on tyrosine and associates with GRB2 in MT-transformed cells. These effects require an intact NPTY motif in MT. SHC immunoprecipitates from MT-transformed cells possess kinase activity that phosphorylates not only SHC and MT but also the 85-kDa subunit of phosphatidylinositol 3-kinase. This result suggests that a complex exists that contains, at a minimum, MT, Src family tyrosine kinases, phosphatidylinositol 3-kinase, and SHC.**

Middle tumor antigen (MT), the principal transforming protein of polyomavirus, has no known intrinsic enzymatic activity. It transforms by associating with and modulating the activities of cellular proteins involved in control of cell proliferation. Among the cellular targets MT binds are the Src family tyrosine kinases $pp60^{c\text{-}src}$ (1), $pp62^{c\text{-}yes}$ (2), and $pp59^{c\text{-}fyn}$ (3). Genetic analysis demonstrated that activation of these kinases is necessary for MT transformation (for review, see refs. 4 and 5). However, two mutants, dl23 and dl1015, activated tyrosine kinases (6, 7) and yet failed to transform, indicating that activation is not sufficient. One explanation that applies to dl23 was found in the phosphorylation of MT. In complexes with tyrosine kinases, MT itself became phosphorylated at Tyr-315 and, to a lesser extent, at Tyr-250 and Tyr-322 (8–10). MTs with mutations at position 315, dl23 (11), and Py1178T (12), or position 250, pT250 (13), were transformation defective. Subsequent research identified a phosphatidylinositol 3-kinase (PI 3-kinase) (14, 15) associated with MT via Tyr-315 and neighboring amino acids (16–18). Immunoprecipitates of dl23 or Py1178T MT were deficient in this activity, providing a biochemical explanation for their transformation defect (15, 19, 20).

However, neither association with tyrosine kinase nor PI 3-kinase could explain a transformation-defective MT mutant carrying Pro-248 → Leu (21). Mutation of amino acids around position 248 generated mutants competent to associate with $pp60^{c\text{-}src}$ and PI 3-kinase and yet transformation-defective. This set of mutants defined a motif in MT, Asn-Pro-Thr-Tyr (or NPTY). Mutation of any of these four amino acids yielded a transformation-defective phenotype (22, 23). Significantly, mutations in NPTY could be rescued by inserting a second NPTY motif at another position in MT. Strikingly, a variant of MT containing two wild-type NPTY motifs yielded foci 5- to 10-fold larger than wild type (23). The NPTY sequence in MT is a member of the more general motif NPXY, first described as important for low-density lipoprotein receptor internalization (24). Although mutation of the NPXY motif of MT might affect its subcellular localization, such a change was not detected (23).

A more general view of cell-growth regulation suggested an explanation for such mutants. Microinjection of anti-Ras antibody blocked growth response to serum (25) and a dominant negative Ras mutant prevented growth of $pp60^{v\text{-}src}$ transformants (26). Ras lies downstream of MT (27, 28), but how MT signals to Ras is unclear. Recently, SHC, a Src homology 2 domain (SH2)-containing protein that couples activated receptor and nonreceptor tyrosine kinases to Ras was cloned (29, 30). SHC protein consists of three overlapping polypeptides of ≈46, 52, and 66 kDa (29, 31). It acts as an intermediate in activation of the GRB2/SOS complex in both epidermal growth factor (EGF)-stimulated and $pp60^{v\text{-}src}$-transformed cells (30, 32). In EGF-stimulated cells, SHC associates with the activated EGF receptor and becomes phosphorylated on tyrosine, creating a motif recognized by the SH2 of GRB2 (33); this leads to formation of a SHC/GRB2/SOS complex thought to activate Ras (32).

Because expression of $pp60^{v\text{-}src}$ induces tyrosine phosphorylation of SHC and subsequent activation of Ras, activated $pp60^{c\text{-}src}$ in complex with MT might do the same. A potential candidate for SHC had been reported in MT immunoprecipitates, a 51-kDa protein of unknown identity (34). Because phosphorylation at Tyr-250 was implicated in transformation, it was tempting to hypothesize that SHC might bind via its SH2 to this site. Our data confirm that SHC does bind to MT in an NPTY-dependent manner.

MATERIALS AND METHODS

Cells and Cell Culture. NIH 3T3 lines expressing wild-type, dl1015 (35), dl23 (11), NG59 (36) MTs or only G418 resistance have been described (37, 38). BALB/3T3 clone A31 cells expressing MT mutants 248-His, 248-His-NPTY, 250-Phe, 250-Phe-NPTY, MT-NPTY have also been described (23). MT point mutants are named by the amino acid mutated and the substitution induced. 248-His-NPTY, 250-Phe-NPTY, and MT-NPTY are NPTY insertion mutants. Cell lines were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM)/10% donor calf serum.

Antibodies, Immunoprecipitation, and Immunoblotting. Rabbit anti-SHC antibody (Upstate Biotechnology) was used for immunoprecipitation of lysates and for immunoblotting (0.5 μg/ml). Mouse anti-GRB2 antibody (1:250; Transduction Laboratories, Lexington, KY) was used for immuno-

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Abbreviations: MT, middle tumor antigen; *P*-Tyr, phosphotyrosine; PI 3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; SH2, Src homology 2 domain; mAb, monoclonal antibody.

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blotting. For most experiments, MT was immunoprecipitated with mouse monoclonal antibody (mAb) PAb750 (from S. Dilworth, Hammersmith Hospital, London) (39). For kinase reactions, rabbit anti-tumor antigen serum (40) was used. For immunoblotting MT, a mixture of mouse hybridoma culture supernatants from mAb PAb750 and F4 (40) (1:80 each) was used. Immunoblotting of phosphotyrosine (*P*-Tyr)-containing proteins was done with mAb 4G10 (41).

Confluent 150-mm dishes of cells were incubated for 20–24 hr in DMEM/0.1% calf serum. Cells were lysed 15 min in 1.0 ml of Nonidet P-40 lysis buffer (1% Nonidet P-40/10% (vol/vol) glycerol/135 mM NaCl/20 mM Tris, pH 8.0/50 mM sodium fluoride/20 mM sodium pyrophosphate/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/aprotinin at 0.03 units/ml). Lysates were scraped and cleared at 13,000 × *g*. SHC was immunoprecipitated from ≈one-tenth of the dish, normalized for total protein content using the Bradford assay (Bio-Rad). For MT immunoprecipitation, extracts were normalized for MT by immunoblotting. Lysates were incubated with rocking at 4°C for 30 min with antibodies and then for 30 min with protein A-Sepharose (for anti-SHC) or protein G-Sepharose beads (Pharmacia) (for mAb PAb750). Immune complexes were washed once with Nonidet P-40 lysis buffer and twice with phosphate-buffered saline (PBS), both containing 1 mM sodium orthovanadate. Washed immunoprecipitates were suspended in sample buffer, heated for 3 min at 95°C, and analyzed by SDS/9% PAGE (42). Immunoblotting was done by standard procedures (43). Washed immunoblots were developed with enhanced chemiluminescence (Amersham).

Kinase Assays. Immune complexes were washed once with Nonidet P-40 lysis buffer, once with PBS, and once with kinase buffer (50 mM Tris·HCl, pH 7.4/10 mM $MnCl_2$). Immunoprecipitates were incubated 15 min at room temperature with 40 μl of kinase buffer (15) containing 20 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq), then washed once, and analyzed by SDS/10.5% PAGE.

RESULTS

Polyomavirus MT Forms Stable Complexes with SHC. Anti-MT immunoprecipitates prepared from cells expressing wild-type MT or from controls were analyzed for SHC by immunoblotting. The major form of SHC, corresponding to the human 52-kDa form, was detected in MT immunoprecipitates from cells expressing wild-type MT (Fig. 1*A*, MT lanes), but not controls (Fig. 1*A*, pLJ and Zip lanes). Association of MT and SHC was confirmed by immunoblotting SHC immunoprecipitates for MT (Fig. 1*B*). Quantitation of the immunoblotting demonstrated that <1% of total 52-kDa SHC species and ≈0.5–2% of total MT are complexed together (data not shown).

MT/SHC Complex Formation Depends on an Intact NPXY Motif in MT. To test the importance of the MT NPXY motif for binding to SHC, transformation-defective MTs containing mutations in either proline (248-His) or tyrosine (250-Phe) of the NPXY motif were examined. Neither bound detectably to SHC (Fig. 1 *A* and *B*). A transformation-defective mutant MT with Asn-247 → Gly was also greatly reduced in SHC binding (data not shown). Two mutants were analyzed to test whether insertion of a wild-type NPTY downstream of the mutated motif, which was known to restore transformation competence, would also restore SHC binding to wild-type levels. This was, indeed, the case for 248-His-NPTY and 250-Phe-NPTY (Fig. 1 *A* and *B*). Finally, MT-NPTY, an NPTY insertion mutant in which a second NPTY was inserted into wild-type MT, also bound SHC (Fig. 1 *A* and *B*).

Although necessary for optimal association with SHC, an intact NPXY in MT was not sufficient. NG59, a MT mutant

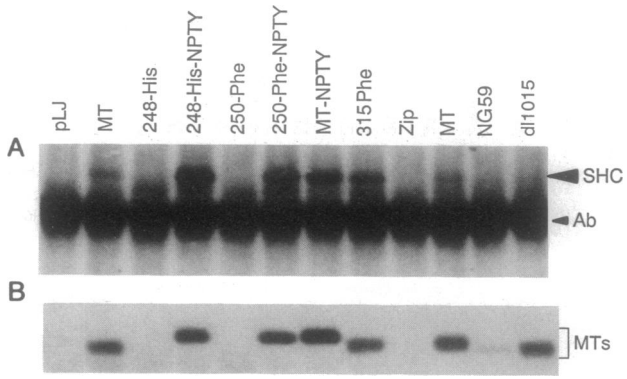

FIG. 1. Association of SHC with MT depends on an intact NPXY motif in MT. (*A*) Lysates were prepared from BALB/3T3 cells expressing only G418 resistance (pLJ), wild-type MT (MT), or various MT mutants (248-His, 248-His-NPTY, 250-Phe, 250-Phe-NPTY, MT-NPTY, and 315-Phe) and from NIH 3T3 cells expressing only G418 resistance (Zip), wild-type MT (MT), or the MT mutants NG59 and dl1015. MT immunoprecipitates were prepared and analyzed by SDS/PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with anti-SHC antibody. In BALB/3T3 cells, the major form of SHC, corresponding to the human 52-kDa form, has an apparent molecular mass of 59 kDa. Positions of this SHC species and of the antibody used for immunoprecipitation are indicated. In this assay, a less intense SHC signal was consistently obtained for dl1015 relative to wild type, whereas, in the assay shown in Fig. 1*B*, dl1015 was consistently wild type for binding SHC. This result may be due to the fact that the deletion in dl1015 is juxtaposed to the probable binding site for PAb750, the mAb (Ab) used for immunoprecipitation in the first assay (39). Identity of the band visible in most lanes of *A*, which migrated between the positions of SHC and antibody, is unknown; it behaved as a nonspecific signal in that it was not consistently seen in different experiments and appeared to some degree in control lanes. (*B*) Anti-SHC immunoprecipitates from the cells described in *A* were analyzed by immunoblotting with anti-MT antibody. Bracket indicates positions of migration of the various MTs.

defective in binding several associated proteins, including pp60$^{c\text{-}src}$, was defective in SHC binding (Fig. 1 *A* and *B*), even though its NPXY motif is intact. The defect of NG59 in associating with pp60$^{c\text{-}src}$ is consistent with the idea that phosphorylation of the NPXY on tyrosine is important for SHC binding.

Mutants at other critical sites in MT were tested to see if sequences besides those around residue 250 might be required for SHC binding. Tyr-315, the principal site of tyrosine phosphorylation, is part of the binding motif for PI 3-kinase. Its mutation to phenylalanine (315-Phe MT) renders the mutant middle defective in PI 3-kinase association (15) and in transformation (12, 13). This MT mutant bound wild-type levels of SHC (Fig. 1 *A* and *B*). Similar results (data not shown) were obtained with dl23 MT which deletes both Tyr-315 and Tyr-322 (11). Transformation-defective dl1015 (35), which deletes a proline-rich sequence in MT, also bound SHC well (Fig. 1*B*).

SHC Is Phosphorylated on Tyrosine in Cells Expressing MTs That Bind SHC. Tyrosine phosphorylation of SHC by activated tyrosine kinases is considered essential for signal transduction from SHC to Ras via GRB2 (30, 31). Analysis of SHC immunoprecipitates free of associated proteins demonstrates increased *P*-Tyr on the 46-kDa and 52-kDa SHC species from cells expressing MTs that bind SHC (Fig. 2*A*). While cells expressing 248-His mutant MT showed a small increase in SHC *P*-Tyr, cells expressing other MTs defective in SHC binding showed little SHC tyrosine phosphorylation.

MT Associated with SHC Is Phosphorylated on Tyrosine. If SHC binds to MT via its SH2 domain, then MT bound to SHC should be phosphorylated on tyrosine. SHC immunoprecip-

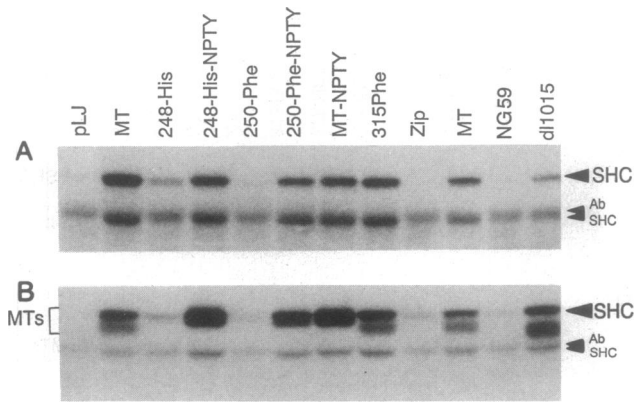

FIG. 2. Tyrosine phosphorylation in SHC immunoprecipitates. (*A*) Phosphorylation of SHC on tyrosine depends on an intact NPXY motif in MT. Lysates from the cells described in Fig. 1 were boiled to dissociate protein complexes. Diluted lysates were immunoprecipitated with anti-SHC antibody, and the immune complexes were immunoblotted with anti-*P*-Tyr antibody. Positions of the 46-kDa and 52-kDa SHC species and of antibody (Ab) are shown. In this particular experiment, the amount of 52-kDa SHC signal in the BALB/3T3 wild-type MT lane (lane 2) exceeds that seen on average. (*B*) MT is phosphorylated on tyrosine in the SHC/MT complex. Unboiled lysates from the cells described in *A* were immunoprecipitated with anti-SHC antibody. Immunoprecipitates were immunoblotted with anti-*P*-Tyr antibody. Positions of MT, SHC, and comigrating antibody (Ab) are indicated.

itates prepared from undenatured lysates to preserve the MT/SHC complex were probed with anti-*P*-Tyr by immunoblotting. Bands comigrating with individual MTs seen in Fig. 1*B* were detected only from cell lines expressing MTs that bind SHC (Fig. 2*B*). Such bands, as expected, vary in size depending on the mutant and, for the more slowly migrating NPTY insert mutants, partially or completely obscure the 52-kDa SHC band (compare Figs. 2*A*, 2*B*, and 1*B*). Importantly, 315-Phe MT lacking Tyr-315 (Fig. 2 *A* and *B*) and dl23MT lacking both Tyr-315 and Tyr-322 (data not shown) were detected by anti-*P*-Tyr. These results are consistent with phosphorylation of Tyr-250 *in vivo*.

Expression of MT that Binds SHC Affects a Significant Fraction of Total 52-kDa SHC in the Cell. Tyrosine phosphorylation of SHC has been reported to correlate with slower migration on gels (44). To assess the over-all state of SHC, cell lysates were immunoblotted with anti-SHC antibody (Fig. 3). In controls, the 52-kDa SHC form was detected as a single band. In cells expressing MTs that complex with SHC, a significant portion migrated more slowly. Cells expressing the insert mutant with two NPTY motifs had >50% of the 52-kDa SHC shifted. Although small amounts were seen in mutants such as 248-His, all MTs defective in binding SHC were clearly defective in inducing the shifted form. Unlike the 52-kDa, the 46-kDa form of SHC did not shift markedly.

SHC Associates with GRB2 in Cells Expressing MTs that Bind SHC. Tyrosine phosphorylation of SHC leads to complex formation between SHC and a 23-kDa protein encoded by the *grb2/sem-5* gene (GRB2) (30). To determine if MT expression induces such complexes, SHC immunoprecipitates were probed with anti-GRB2 antibody. Although a low level of SHC/GRB2 complex was detectable in all cells, the presence of a MT that binds SHC caused a large increase in these complexes (Fig. 4). GRB2, like SHC, was also found in MT immunoprecipitates (data not shown). Because much more GRB2 was immunoprecipitated with anti-SHC than with anti-MT, most GRB2/SHC complexes are not associated with MT.

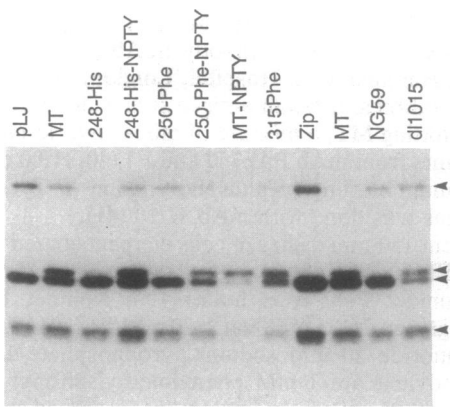

FIG. 3. SHC is shifted in gel mobility in response to MT expression. Lysates from the cells described in Fig. 1 were analyzed by SDS/PAGE, transferred to nitrocellulose, and probed with anti-SHC antibody. Positions of the various forms of SHC (46-, 52-, and 66-kDa) are indicated by arrowheads. Although barely visible, a small amount of shifted 52-kDa SHC is consistently seen for 248-His MT. Even on long exposure, no shifted form of the 46-kDa SHC can be detected.

A MT Complex Exists Containing Both PI 3-kinase and SHC. The results with mutants NG59 and 250-Phe support the hypothesis that a tyrosine kinase must associate with MT and phosphorylate Tyr-250 to bind SHC efficiently. SHC immunoprecipitates from MT-expressing cells should therefore contain kinase activity. To test this, anti-SHC immunoprecipitates from several cell lines were incubated with [γ^{32}-P]ATP. Kinase activity was detected in SHC immunoprecipitates from wild-type and MT-NPTY MT-expressing cells but not from cells expressing no MT or the 250-Phe MT mutant (Fig. 5*A*). Thus, MT/SHC complex formation results in SHC-associated kinase activity. This activity(s) phosphorylated both tyrosine and serine residues in the immune complex (data not shown). The 85-kDa band phosphorylated in SHC immunoprecipitates was confirmed by peptide mapping to be the 85-kDa subunit of PI 3-kinase, and PI 3-kinase activity was found in the corresponding immune complex (data not shown).

DISCUSSION

This report shows by coimmunoprecipitation that MT binds the protooncogene SHC. The NPXY of MT is critical for this interaction. Mutation in this motif greatly diminishes binding. Reinsertion of an NPTY between residues 282 and 283, yielding the sequence shown in Table 1, restores it. Mutations in the NPXY motif can strongly reduce the transforming ability of MT. Reinsertion restores it. Because SHC association parallels transforming ability, it is reasonable to postulate a direct role for SHC in transformation by MT.

Three species of SHC are known. Although these data primarily concern the major 52-kDa form, at least some of the

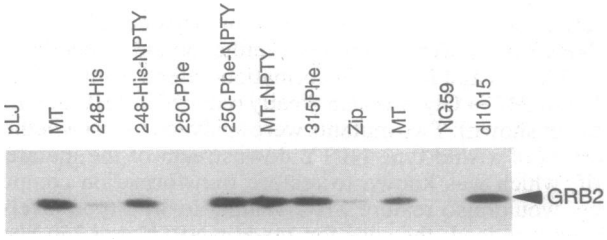

FIG. 4. Induction of SHC/GRB2 association by MT requires intact NPXY motif. Lysates from the same cells described in Fig. 1 were immunoprecipitated with anti-SHC antibody. Proteins in the immune complexes were immunoblotted with anti-GRB2. Arrowhead, position of GRB2.

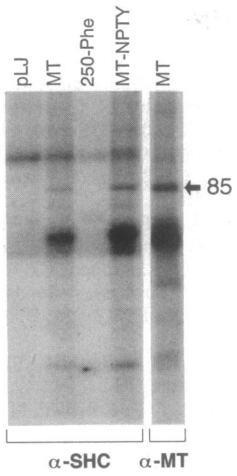

FIG. 5. Protein kinase activity is associated with SHC in MT-expressing cells. Lysates from BALB/3T3 cells expressing only G418-resistance, wild-type MT, 250-Phe, or MT-NPTY were immunoprecipitated with anti-SHC antibody. In parallel, lysate from the wild-type MT-expressing cells was also immunoprecipitated with anti-tumor antigen antibody. The immune complexes were incubated with [γ-^{32}P]ATP. Phosphoproteins were visualized by SDS/PAGE and autoradiography. The MT lane is from a shorter exposure of same gel than the other lanes. Arrow, position of p85 subunit of PI 3-kinase.

46-kDa form was seen in MT immunoprecipitates (data not shown). This is consistent with its observed tyrosine phosphorylation in MT-expressing cells. Only a small fraction of the 46- and 52-kDa forms (<1%) is associated with MT. Whether the least abundant 66-kDa SHC form associates is unclear. Its level varies among cell lines (see Fig. 3) in a manner still to be explained.

In response to MT expression, SHC exhibits the hallmarks of activation: tyrosine phosphorylation and association with GRB2. Immunoblotting shows that the 46- and 52-kDa species are phosphorylated on tyrosine, and a large fraction of the 52-kDa form is shifted in mobility. SHC binds GRB2 when MT is expressed. Both the amount of SHC associated with GRB2 and the amount shifted in mobility are much greater than that in the MT complex. Because these effects require the ability of MT to complex with SHC, the MT complex appears to affect SHC in a "catalytic" manner. Alternatively, binding of SHC to MT may trigger a signal that results in widespread modification of SHC.

How does SHC binding relate to MT transformation? Signaling via p21ras is essential for MT (27, 28). Because SHC functions in Ras signaling pathways in other systems, SHC probably participates in this process in MT transformation. The SHC/GRB2 complex interacts with SOS, an exchange factor for Ras. Preliminary results indicate this interaction also occurs (data not shown). MT may also send other signals via SHC. For instance, the NPXY motif was first described in the low-density lipoprotein receptor, where it functions in receptor internalization. Because membrane trafficking involves a variety of small G proteins, the NPTY motif in MT, and hence SHC, may also be involved in trafficking. Currently, little data exist on the trafficking of MT. Finally, the signal originating from the NPXY motif may be more complex; the data presented here do not exclude the possibility that an additional protein(s) may bind at this same site.

What is the molecular nature of the interaction between MT and SHC? The simplest hypothesis would be that the SHC SH2 domain binds to tyrosine-phosphorylated MT. Certainly MT phosphorylation is connected to binding. Mutation of the phosphorylation site at 250 abolishes SHC binding. Preliminary data indicate that MT binds the SHC SH2 domain *in vitro*. This interaction is inhibited by phosphorylated peptide-containing

Table 1. Examples of receptors and oncogenes containing NPXY motifs

Receptor or oncogene	Position of tyrosine	NPXY motif in context
Wild-type MT	250	SLLSNPTYSVMR
dl8 MT	250	SLLSNPTYSVPM
MT-NPTY (insert location)	286	LVLLNPTYLSPM
TrkA (human)	490	HIIENPQYFSDA
TrkB (mouse)	515	PVIENPQYFGIT
TrkC (pig)	516	PVIENPQYFRQG
Trk (rat)	499	HIMENPQYFSDT
erbB-2 (human)	1196	GAVENPEYLTPQ
erbB-2 (human)	1248	PTAENPEYLGLD
neu (rat)	1201	GAVENPEYLVPR
neu (rat)	1253	PTAENPEYLGLD
EGFR (human)	1086	GSVQNPVYHNQP
EGFR (human)	1114	TAVGNPEYLNTV
EGFR (human)	1148	ISLDNPDYQQDF
EGFR (chicken)	1149	TAVDNPEYLNTN
EGFR (chicken)	1183	INLDNPDYQQDF
EGFR (chicken)	1208	PAAENPEYLRVA
EGFR (*Drosophila melanogaster*)	1228	VSVDNPEYLLNA
Melanoma receptor (fish)	1077	SDIYNPNYEDLT

Examples of NPXY motifs in MTs and receptors. Three different transforming alleles of MT and some examples of tyrosine kinase receptors possessing NPXY motifs are listed. dl8 MT is a MT deletion mutant missing aa 252–283 (45). EGFR, EGF receptor. Position numbers listed for chicken and *D. melanogaster* EGFRs are for receptor precursors. Note that homology extends beyond the simple NPXY. Underlined residues in the proposed binding sites from several MTs and Trks represent possible extended consensus binding residues upstream of NPXY. Striking homology exists between MT NPTY insertion mutants and human erbB-2 (VLLNPTYLSP vs. AVENPEYLTP) and between dl8 MT and the rat erbB-2 equivalent, neu (LLSNPTYSVP vs. AVENPEYLVP). These homologous sequences in human erbB-2 and rat neu surround Tyr-1196 and Tyr-1201, respectively. The EGFR family generally has multiple NPXY motifs. In such receptors, ones with extended homology may represent likely SHC-binding sites.

sequence surrounding Tyr-250. The double mutants, in which a new NPTY is inserted into a 248-His or 250-Phe mutant background, have regained the ability to bind SHC. Phosphopeptide analysis of MT labeled *in vitro* showed that tyrosine at the new position was phosphorylated (data not shown).

What is the significance of the critical NPXY motif? The possibility that upstream sequences are required for phosphorylation is excluded by the observation that the 248-His mutant is phosphorylated like wild type (data not shown). Most work on the context of the *P*-Tyr with respect to SH2 recognition has focused on sequences C-terminal to the *P*-Tyr (33, 46–50). Thus, for example, SH2s of PI 3-kinase prefer pYMXM sequences consistent with the pY^{315}MPM of MT. Our data suggest the possible involvement of upstream sequences in the case of SHC. The amino acid sequence in wild-type MT subsequent to *P*-Tyr-250 is SVM. However, in the mutant inserting the motif between residues 282 and 283, the trailing sequence is LSP, which differs significantly (as is the sequence after the tyrosine involved in SHC binding in the human NGF receptor; see below). This result is consistent with the genetic analysis showing residues upstream of the *P*-Tyr as critical for the interaction. Some evidence in other systems (51, 52) is emerging, consistent with this view. However, it remains highly likely that downstream sequences also contribute. An alternative, but more exciting possibility than the simple model, is that interaction of MT with SHC arises from a novel molecular mechanism.

Whatever the details of the interaction between SHC and the NPXY motif, SHC may also bind some other proteins that have NPXY motifs. Table 1 lists some of hundreds of polypeptides that contain this motif. For those that have intrinsic

or associated tyrosine kinase activity, their NPXY motif(s) might function in binding SHC. Data on the nerve growth factor tyrosine kinase receptor, Trk, provide a known example; mutation of tyrosine in the sequence NPQY blocks binding of SHC (54). Two other activated receptor tyrosine kinases, EGF receptor and erbB-2, bind SHC (29, 31). In both cases, the SHC SH2 domain was sufficient for association. For erbB-2, the binding site was localized to the C-terminal region containing the autophosphorylation sites, including two with the sequence NPEY (Table 1). Simultaneous mutation of all five of these sites abolished SHC binding.

The MT complex resembles an activated growth factor receptor. SHC is the third SH2-containing protein known to complex with MT. Others probably remain to be discovered. In some MT complexes, PI 3-kinase and SHC appear to be on the same MT molecule. With growth factor receptors it has sometimes been difficult to see effects on mitogenesis by mutating any one of several SH2-binding sites. For instance, the phospholipase Cγ- and SHC-binding sites in the nerve growth factor receptor are individually dispensable for nerve growth factor-mediated neurite outgrowth. MT-mediated transformation provides a sensitive system for studying signaling. Mutation of either the NPXY or the YMPM motif used for PI 3-kinase binding severely diminishes transformation function (12, 23). The sensitivity of MT to mutation may result from the small number of activated MT molecules in a cell. Additional insight into transformation should come from the study of MT mutants like dl1015, which are transformation defective but interact with all known host proteins (34, 38), including SHC.

Note: While this work was under review, similar findings were reported by Dilworth *et al.* (53).

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