Identification of Src Phosphorylation Sites in the Catenin p120ctn*

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p120-catenin (p120ctn) interacts with the cytoplasmic tail of cadherins and is thought to regulate cadherin clustering during formation of adherens junctions. Several observations suggest that p120 can both positively and negatively regulate cadherin adhesiveness depending on signals that so far remain unidentified. Although p120 tyrosine phosphorylation is a leading candidate, the role of this modification in normal and Src-transformed cells remains unknown. Here, as a first step toward pinpointing this role, we have employed two-dimensional tryptic mapping to directly identify the major sites of Src-induced p120 phosphorylation. Eight sites were identified by direct mutation of candidate tyrosines to phenylalanine and elimination of the accompanying spots on the two-dimensional maps. Identical sites were observed in vitro and in vivo, strongly suggesting that the physiologically important sites have been correctly identified. Changing all of these sites to phenylalanine resulted in a p120 mutant, p120–8F, that could not be efficiently phosphorylated by Src and failed to interact with SHP-1, a tyrosine phosphatase shown previously to interact selectively with tyrosine-phosphorylated p120 in cells stimulated with epidermal growth factor. Using selected tyrosine to phenylalanine p120 mutants as dominant negative reagents, it may now be possible to selectively block events postulated to be dependent on p120 tyrosine phosphorylation.

Cadherins constitute a major superfamily of transmembrane cell-cell adhesion receptors present in most adherent cell types. The extracellular domains mediate adhesion between adjacent cells through calcium-dependent homophilic interactions. The cytoplasmic domains interact with a group of proteins called catenins (e.g. p120ctn, a-catenin, and b-catenin), which regulate cadherin interactions with the actin cytoskeleton. b-Catenin (or g-catenin/plakoglobin) (1) and p120 (2, 3) are Armadillo (Arm) repeat domain proteins (5-7), respectively. b-Catenin is hardwired to the actin cytoskeleton through its interaction with a-catenin (8, 9), which binds to F-actin directly (10) or through accessory proteins such as a-actinin (11, 12). p120, on the other hand, does not interact with a-catenin (13) and is thought to modulate the actin cytoskeleton indirectly (for review see Ref. 14), possibly through regulation of Rho family GTPases (15-17).

Src and receptor tyrosine kinases are frequently up-regulated or activated in human cancers, and multiple tyrosine kinases (e.g. Src, Er, epidermal growth factor receptor, colony-stimulating factor receptor, platelet-derived growth factor receptor, and nerve growth factor receptor) (18-22) and phosphatases (e.g. SHP-1, SHP-2, PTP-µ, PTP-K, PTP-LAR, and PTP-1B) (23-27) have been directly or indirectly linked to cadherin complexes. In general, cadherin-mediated adhesion is compromised in cells expressing activated tyrosine kinases, but the extent to which direct phosphorylation of the catenins contributes to defects in cadherin function is unclear.

Studies of endogenous Src family proteins suggest both positive and negative roles in adhesion. In one study, cell adhesion defects were observed in keratinocytes and epidermal cells from Fyn-deficient mice or from mice with double knockouts of Src and Fyn, suggesting that the activities of Src kinases are necessary for normal cell-cell adhesion (28). In contrast, another study showed that inhibition of the catalytic activity of the Src kinases promotes the stability of cadherin-dependent cell-cell contacts (29), suggesting that Src kinase activity is normally required for junction disassembly. The latter result is consistent with data from oncogene-transformed or mitogen-stimulated cells, where increased tyrosine kinase activity is associated with weak junctions (for review see Ref. 30). The simplest explanation for these apparently contradictory findings is that controlled tyrosine kinase activity is necessary for both assembly and disassembly of adherens junctions, depending on regulatory influences that are poorly understood.

It has been suggested that constitutive tyrosine phosphorylation of p120 and b-catenin contributes to defects in cadherin-mediated adhesion in Src-transformed cells (for review see Ref. 30). Although evidence suggests that these defects are independent of b-catenin tyrosine phosphorylation (31), a role for p120 tyrosine phosphorylation has not been excluded. Indeed, mutation of p120 tyrosine residue 217 can partially reverse the Src-induced defects in cadherin function (32) in L-cells. An unresolved problem, especially in Src-transformed cells, is how to separate the effects of p120 tyrosine phosphorylation from the simultaneous effects induced by tyrosine phosphorylation of other substrates. For example, most prominent Src substrates (e.g. tensin, focal adhesion kinase, Crk-associated substrate, actin filament-associate protein, cortactin, ezrin-radin-moerin proteins, p120, b-catenin, etc.) are proteins that affect the organization of the actin cytoskeleton (33). Thus, it is possible that constitutive phosphorylation of these proteins affects cadherin function indirectly by negatively affecting the underlying actin framework that anchors the cadherin complex. Therefore, although a vast literature correlates cadherin...
malfunction to unscheduled tyrosine phosphorylation of the catenins, direct mechanistic evidence of a role for p120 phosphorylation is lacking.

To directly address the role of p120 tyrosine phosphorylation in cadherin function, we aim to generate dominant negative p120 Tyr → Phe mutant proteins that are selectively unable to be phosphorylated at key tyrosine residues. Here, as a first step toward this goal, we have employed two-dimensional tryptic mapping methods to identify all major sites of Src-induced p120 tyrosine phosphorylation. A method was developed to tyrosine phosphorylate p120 to high stoichiometry using a modification of the in vitro Src kinase assay. Deletion mapping revealed that virtually all p120 tyrosine phosphorylation occurs in a region amino-terminal to the first Arm repeat. Thus, we have named this region the p120 phosphorylation domain and suggest that it is involved in the regulation of p120 function. To confirm the identity of each site, individual candidate tyrosine residues were mutated to phenylalanine and assayed for elimination of the accompanying spot on the two-dimensional tryptic phosphopeptide map. Importantly, p120 tryptic maps from in vitro labeling experiments were nearly identical to p120 maps generated by [32P]orthophosphate labeling in vivo, indicating that we have indeed mapped the sites that are likely to be relevant in vivo. Eight sites were identified, and changing these sites to phenylalanine resulted in a p120 mutant (p120–8F) that could not be phosphorylated efficiently by Src and failed to interact with SHP-1, a phosphatase known to bind p120 in a tyrosine phosphorylation-dependent manner. Using mutants with individually altered tyrosine phosphorylation sites, it may now be possible to distinguish the specific effects of p120 phosphorylation from effects driven by phosphorylation of other substrates induced by Src and receptor tyrosine kinases.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—Cells were cultured in Dulbecco’s modified Eagle’s medium containing l-glutamine (Life Technologies, Inc.), 10% fetal bovine serum (HyClone), and 1% penicillin-streptomycin (Life Technologies, Inc.). Cos-7 (African green monkey kidney) were obtained from American Type Culture Collection and from Dr. Steven Hanks (Vanderbilt University); HEK 293 (human embryonic kidney) cells were obtained from the American Type Culture Collection. Transient transfections were performed with SuperFect transfection reagent (Qiagen) according to the manufacturer’s directions, except that DNA-SuperFect complexes were allowed to form for 15 min prior to the addition to cells. In co-transfection experiments, equal micromolar amounts of each plasmid were transfected (5 μg of total plasmid/60-mm dish). Transfections were carried out with 5 μl of SuperFect/1 μg of DNA for Cos-7 cells and 6 μl of SuperFect/1 μg of DNA for 293 cells. Experiments on transfected cells were performed 1 day post-transfection.

Transfected constructs include RcRSV/mp120–1A (wild type and Tyr → Phe point mutante), RcRSV/mp120–1N Δ1-157, RcCMV/mp120–1N Δ29-233, RcCMV/mp120–1N Δ233-387, RcRSV/Src527F, and pcDNA3.1-c-Src (K430R).

Cell Lysis, Immunoprecipitation, and Western Blotting—Cell lysis, immunoprecipitation, and Western blotting procedures have been described in detail previously (13). Briefly, cells were washed one time in phosphate-buffered saline (10 mM phosphate, pH 7.4, 150 mM NaCl) and then lysed in 1 ml of ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA) for 5 min at 0 °C. Lysates were clarified by centrifugation for 5 min. Monoclonal antibody (mAb) was added to the supernatant and incubated for 1 h at 4 °C with end-over-end rotation. For phosphorylation studies, the mAb 8D11 was added to lysates and incubated for an additional 1 h at 4 °C. Immunoprecipitates were washed five times with lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled for 3 min. Denatured proteins were separated by SDS-polyacrylamide gel electro-
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29 °C for 14 h. Bacteria were harvested by centrifugation and resuspended in detergent buffer (10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4/150 mM NaCl/0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM β-mercaptoethanol, and 1 mM EDTA. Bacterial suspensions were sonicated on ice, and lysate was clarified by centrifugation (14,000 × g, 10 min) at 4 °C. GST proteins were immobilized by incubating the clarified lysate with 250 μl of glutathione-Sepharose beads (Sigma) for 1 h at 4 °C with end-over-end rotation. The beads were washed twice with detergent buffer and twice with phosphate-buffered saline and resuspended to a final volume of 500 μl to generate a 50:50 bead slurry for pull-down experiments. GST proteins were gel-quantified relative to known protein loading standards.

GST Pull-down of Tyrosine-phosphorylated p120—Cos-7 cells were transiently co-transfected with Src527F and murine p120, and in vitro kinase assays were performed with the immunoprecipitated protein, as described above. Kinase assays were done in the presence of 20 μM ATP (Sigma) rather than with [γ-<sup>32</sup>P]ATP. Following kinase assays, beads were washed twice with RIPA and then boiled for 5 min in denaturation buffer (50 mM HEPES, pH 7.5, 1% SDS, 1% β-mercaptoethanol). For samples cooled to ambient temperature, an aliquot of sample was reserved for Tyr(P) and p120 Western blotting to determine the relative amount of p120 present in each pull-down reaction. Then 600 μl of 0.5% Triton X-100 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM EDTA, and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) was added to each sample to dilute the SDS to less than 0.05%. The beads were discarded from samples, and the proteins were allowed to partially renature in this solution for 30 min at 4 °C. Glutathione-Sepharose beads loaded with GST or SHP-1 SH2 domain GST fusion protein were added, and samples were incubated at 4 °C for 1.5 h with end-over-end rotation. Then beads were washed three times with 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100 and boiled in 2× Laemmli sample buffer for 3 min.

RESULTS

Characterization of p120 Phosphorylation in Vivo and in Vitro—To determine the extent of total p120 phosphorylation under steady state conditions in vivo, murine p120 was transiently overexpressed and orthophosphate-labeled in rapidly growing 293 cells. Two-dimensional trypsin phosphopeptide mapping of p120 resolved two peptides that were robustly phosphorylated, as well as a complex array of minor phosphopeptides (Fig. 1A). Phosphoamino acid analysis of the total protein revealed that a majority of the phosphorylation was on serine, with a relatively small amount of phosphorylation on tyrosine. Phosphoamino acid analysis of the two major peptides (Fig. 1A, peptides C and H) indicated that the phosphate was on serine (data not shown). Because p120 was initially discovered as a major in vivo substrate for Src tyrosine kinase, we overexpressed p120 together with Src527F, a constitutively active Src mutant. Overexpression of Src527F lead to a sharp increase in the phosphotyrosine content of p120, as determined by phosphoamino acid analysis (Fig. 1B, compare − and +Src panels), producing a unique array of p120 phosphopeptides (Fig. 1A, panel ii). Because of the high stoichiometry of tyrosine phosphorylation, phosphoserine-containing peptides were no longer detectable at optimal exposure times for phosphotyrosine detection. Five-minute pervanadate treatment prior to cell lysis was required for optimal detection of phosphotyrosine. Five-minute pervanadate treatment prior to cell lysis was required for optimal detection of phosphotyrosine, phosphothreonine, and phosphotyrosine are circled. C, direct p120 phosphorylation by Src in vitro. Immune complexes containing the indicated (+/−) combinations of p120 or Src were subjected to in vitro kinase assay, separated by SDS-polyacrylamide electrophoresis, and visualized by autoradiography. kd represents kinase-dead Src. p120 phosphorylation occurs only in the presence of both p120 and kinase active Src molecules.

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rylation—Initially, a panel of p120 deletion mutants was assayed to identify regions of p120 that could be phosphorylated by Src. Only deletions in the amino terminus of p120 affected the phosphorylation pattern on tryptic maps (Fig. 2), in agreement with previous data indicating that most of the major phosphorylation sites were contained in an 18-kDa amino-terminal fragment generated by cleavage with the protease Endolysine C. Deletion of the first 157 amino acids eliminated the major phosphopeptides 6, 9, and 10 (Fig. 2). Deletion of amino acids 29–233 eliminated one additional peptide, peptide 7, suggesting that three of the major phosphopeptides originated from amino acids 29–157, whereas another peptide lay between amino acids 158 and 233. Finally, deletion of amino acids 233–387 eliminated the remaining major phosphopeptides, 1–5 and 8, suggesting the location of the remaining sites. In addition, the latter deletion eliminated peptide 7, and a new peptide designated peptide A was observed. These experiments place all of the major sites within the amino-terminal 387 residues.

Partial tryptic digestion frequently results in overlapping peptides containing the same phosphorylated residue. To determine which of the phosphopeptides shared amino acid sequence similarity, individual peptides were isolated and treated with hydrochloric acid to detect similarities in the pattern of partial hydrolysis products that are characteristic of each peptide (Fig. 3). These "peptide fingerprints" showed a relationship between peptides 1–3 (Fig. 3A), peptides 4 and 5 (Fig. 3B), peptides 9 and 10 (Fig. 3C), and peptides 7 and A (not shown). Furthermore, each of these sets of peptides behaved similarly when oxidized with performic acid (not shown).

Based on deletion mapping, peptide fingerprinting, and performic acid oxidation data (not shown), the search was narrowed to specific candidate residues. To definitively identify each site, suspected tyrosines were individually mutated to phenylalanine and re-evaluated by in vitro kinase assay and two-dimensional tryptic mapping (Fig. 4). Mutation of tyrosines 112, 228, and 280 to phenylalanine resulted in elimination of phosphopeptide spots 6, 7, and 8, respectively. Mutation of tyrosine 96 eliminated spots 9 and 10, whereas mutation of tyrosine 257 eliminated spots 4 and 5. The latter results confirm the partial hydrolysis fingerprinting data, which sug-

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2 A. B. Reynolds, unpublished observations.
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Fig. 4. Identification of major phosphorylation sites by mutational analysis in vitro (left panels) and in vivo (right panels). Left panels, p120 mutants were labeled by in vitro Src kinase assay and subjected to two-dimensional tryptic mapping. Exposure times were: 20,000 cpm each; all exposed for 1 h, except for 112F and 228F, which were exposed for 2.5 and 1.5 h, respectively. Right panels, p120 mutants were transiently co-expressed in 293 cells with Src527F, orthophosphate-labeled in vivo, and subjected to two-dimensional mapping. Exposure times were: Wild type, 112F, and 228F, 820 cpm, 2.3 days; 96F, 257F, and 280F, 700 cpm, 4.5 days. The data were compiled from independent, representative experiments. X, sample origin. The horizontal arrow indicates the direction of electrophoresis at pH 1.9 (toward the cathode). The vertical arrow indicates the direction of chromatography.

Gestated that each of these pairs of spots arose from partial tryptic digestion of the same sequence. Identification of Tyr<sup>228</sup> in peptide 7 is in accordance with the deletion analysis, which indicated that peptide 7 resided between amino acids 158 and 233. The unique peptide A present in p120Δ233–387 apparently results from the new fusion peptide formed by the deletion.

In addition, each p120 point mutant was transiently co-transfected with Src527F into 293 cells and labeled in vivo by the addition of [32P]orthophosphate to the cell culture medium. Note that the transfected p120 mutants were generated from murine p120 and can therefore be selectively immunoprecipitated from human 293 cells using p120 mAb 8D11, which recognizes murine but not human p120. Two-dimensional tryptic maps generated by directly immunoprecipitating the transfected p120 from these cells demonstrated that the major peptides that were lost corresponded exactly to those identified in vitro (Fig. 4, compare in vitro and in vivo columns). These maps of in vivo labeled p120 also revealed occasional unique phosphopeptides that were not readily reproducible.

Tryptic mapping of point mutants initially failed to lead to identification of peptides 1, 2, and 3, which had been shown to be related to each other by partial hydrolysis fingerprinting (Fig. 3). To identify the phosphorylated tyrosines in these peptides, we explored the possibility that these spots arose from a single peptide that was phosphorylated at multiple sites. Because increased phosphorylation (and therefore, net negative charge) of a peptide decreases both electrophoretic potential and hydrobicity, single peptides phosphorylated at multiple sites are predicted to migrate on a diagonal with positive slope. This pattern characterized the migration of peptides 1, 2, and 3. Peptide 3 migrated toward the cathode, indicating a low charge-to-mass ratio, and was relatively hydrophilic. Mutating Tyr<sup>291</sup>, Tyr<sup>296</sup>, or Tyr<sup>302</sup>, which lie in a single predicted tryptic fragment, each resulted in elimination of peptide 3 while concomitantly increasing the hydrobicity of peptides 1 and 2 (Fig. 5, 296F). Further mutation of both Tyr<sup>296</sup> and Tyr<sup>302</sup> resulted in elimination of both peptides 2 and 3, and a further increase in hydrobicity of peptide 1 (Fig. 5, 296/302F). These results suggest that peptides 1, 2, and 3 represent singly, doubly, and triply phosphorylated variants, respectively, of the same peptide, containing Tyr<sup>291</sup>, Tyr<sup>296</sup>, and Tyr<sup>302</sup>. Indeed, triple mutation of each of these putative phosphorylation sites completely eliminated peptides 1, 2, and 3 without detectably affecting the migration of any other phosphopeptides (Fig. 5, 291/296/302F). This result was confirmed by in vivo orthophosphate labeling of ectopic murine p120 containing Y291F/Y296F/Y302F mutations (not shown). The results of the point mutation analyses and the final assignments of each site are summarized in Table I.

Mutational Analysis of SHP-1 Binding to Tyrosine-phosphorylated p120—SHP-1 is an SH2 domain-containing nonreceptor tyrosine phosphatase shown previously to interact with tyrosine-phosphorylated p120 (37). In theory, our mapping data should allow the identification of specific p120 tyrosine residues that mediate the interaction. To test this hypothesis and confirm the relevance of the identified sites, we performed a series of GST-SHP-1 pull-down experiments. GST was fused to the tandem SH2 domains of SHP-1 (generating GST-SHP-SH2) and used to pull down p120 mutant proteins containing specific tyrosine to phenylalanine mutations.

Consistent with previous data, p120 that was phosphorylated in vitro by Src associated tightly with the GST-SHP-SH2 probe but not with GST alone (Fig. 6). To test whether this association was dependent on p120 tyrosine phosphorylation, we repeated the pull-down assays with a p120 mutant where each of the eight identified tyrosine phosphorylation sites was changed to phenylalanine (i.e. p120–8F). The p120–8F mutant failed to interact with GST-SHP-SH2 (Fig. 6A, compare lanes 1–3). As expected, tyrosine phosphorylation of p120–8F was nearly undetectable on anti-phosphotyrosine Western blots (Fig. 6C, lane 3). These data further confirm that the p120-SHP-1 interaction is tyrosine phosphorylation-dependent and suggest that the identified sites of p120 phosphorylation are sufficient to mediate the interaction with the SHP-1 SH2 domain.

On the other hand, no single p120 Tyr → Phe mutation was sufficient to significantly decrease the p120 SHP-1 SH2 interaction (data not shown). One possibility is that each of the two SH2 domains of SHP-1 interacts simultaneously with unique sites in p120. Alternatively, individual SH2 domains could...
main a “trapping mutation,” C455S, which renders the phosphatase unable to release its target (not shown). Therefore, although we were able to demonstrate dependence upon the tyrosine phosphorylation sites we identified, we were unable to define specific phosphorylation events that mediate the p120-SHP-1 interaction.

**DISCUSSION**

Although Src- and receptor-induced tyrosine phosphorylation of p120 has been demonstrated in many systems, it is unclear how this modification affects p120 or cadherin function. A major obstacle is the fact that these kinases regulate multiple substrates simultaneously, making it difficult to distinguish the effects of one modified substrate from another. Moreover, p120 is phosphorylated at multiple sites, each of which might have different functions. To pinpoint the specific effects of p120 in these processes, we aim to create specific dominant negative p120 mutants that lack the ability to be phosphorylated at key sites. As a first step toward this goal, we have identified the major Src-induced tyrosine phosphorylation sites on p120 and generated a panel of tyrosine to phenylalanine p120 mutants, including one in which all eight sites are mutated simultaneously (p120–8F). The latter mutant may be particularly useful in screens designed to initially identify activities dependent on p120 tyrosine phosphorylation. Indeed, p120–8F was not significantly tyrosine-phosphorylated in *in vitro* Src kinase assays, and it failed to interact with SHP-1, a nonreceptor tyrosine phosphatase shown previously to bind tyrosine-phosphorylated p120 via the SHP-1 amino-terminal SH2 domain (37).

Fig. 7 shows the location of all phosphorylated and unphosphorylated p120 tyrosine residues. Of the 30 tyrosines, 11 are located carboxyl-terminal to the first Arm repeat, and none of these phosphorylated by Src. Thus, the Arm domain does not appear to be an important target for phosphorylation. Of the remaining 19 tyrosines located amino-terminal to the first Arm repeat, eight were phosphorylated. Six of these occur in a relatively short segment between residues 228 and 302, an apparent phosphorylation domain. This region also contains the majority of p120 serine phosphorylation sites, suggesting that most regulatory modification of p120 function is likely to occur through serine and tyrosine phosphorylation within this domain. Only two sites, tyrosines 96 and 112 occurred outside this region; residue 96 is present in isoform 1 (the most common form in mesenchymal cells) but absent from isoform 3 (the most common form in epithelial cells), suggesting a potential role in cell motility. Of the eight identified sites, only tyrosine 291 is not conserved among human, mouse, and chicken (phenylalanine in chicken p120).

Although the clustering of sites within the p120 phosphorylation domain was striking, it was apparently not the only factor influencing the choice of a particular residue by Src because many of the tyrosine residues in this region clearly were not phosphorylated. Consensus motifs for preferential phosphorylation by Src and receptor tyrosine kinases have been identified in *in vitro* by screening peptide libraries (38). The predicted motifs based on such studies include 1) acidic residues favored but not necessary in the −3 and −2 positions upstream of the phosphorylation site, 2) a β-branched residue (isoleucine, valine) in the −1 position, 3) a small or acidic amino acid in the +1 position, and 4) a large hydrophobic amino acid in the +3 position (38). Indeed, aside from the single exception of tyrosine 662, all of the p120 tyrosine motifs that contained at least two of the four parameters described above were in fact identified as phosphorylation sites in our study. Of these, the most con-

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**FIG. 5. Identification of peptides 1–3 as a single triply phosphorylated peptide.** Wild type or mutated p120 (panels 296F, 296/302F, and 291/296/302F) was phosphorylated *in vitro* by Src527F and subjected to two-dimensional tryptic mapping. Mutation of Tyr^296^ to Phe (panel 296F) resulted in increased hydrophobicity of peptides 1 and 2, resulting in an upward shift to positions 1 and 2, respectively. Additionally, peptide 3 was no longer phosphorylated. The circles show the normal positions of these peptides on a wild type p120 map. Mutation of both Tyr^296^ and Tyr^302^ to Phe (panel 296/302F) further increased the hydrophobicity to peptide 1, resulting in an additional upward shift to position 1, and peptide 2 was no longer phosphorylated. Mutation of Tyr^291^, Tyr^296^, and Tyr^302^ to Phe (panel 291/296/302F), which are predicted to lie in a single tryptic peptide, completely abolished phosphorylation of peptides 1, 2, and 3, indicating that these peptides represent a single triply phosphorylated peptide. x, sample origin. The horizontal arrow indicates the direction of electrophoresis at pH 1.9 (toward the cathode). The vertical arrow indicates the direction of chromatography. Exposure time was: 10,000 cpm, 3 h.
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**TABLE I**

| Peptide | Phosphorylated tyrosines(s) | Tryptic fragment |
|---------|-----------------------------|-----------------|
| 1       | Single PO<sub>4</sub>; 291, 296, 302 | 288SMGY<sup>217</sup>DDDL<sup>218</sup>Y<sup>296</sup>GMMSD<sup>297</sup>GTAR<sup>306</sup> |
| 2       | Double PO<sub>4</sub>; 291, 296, 302 | 288SMGY<sup>217</sup>DDDL<sup>218</sup>Y<sup>296</sup>GMMSD<sup>297</sup>GTAR<sup>306</sup> |
| 3       | Triple PO<sub>4</sub>; 291, 296, 302 | 288SMGY<sup>217</sup>DDDL<sup>218</sup>Y<sup>296</sup>GMMSD<sup>297</sup>GTAR<sup>306</sup> |
| 4 and 5 | 257                      | 254QDV<sup>255</sup>GRFY<sup>256</sup> |
| 6       | 112                     | 102QEGQIVETY<sup>207</sup>EEDEPGAMSVVSVETTDGTR<sup>215</sup> |
| 7       | 228                     | 216HYEDGYPGSDDNY<sup>226</sup>GSLSR<sup>233</sup> |
| 8       | 280                     | 273FHEPFY<sup>276</sup>GLEDQDR<sup>283</sup> |
| 9 and 10 | 96                     | 8LNPQDHHLLY<sup>87</sup>STIPR<sup>91</sup> |

**Fig. 6.** The SHP-1 SH2 domains interact with p120 in a phosphotyrosine-dependent manner. Wild type p120 (isoform 1A) or p120 containing Tyr→Phe point mutations (see below) was phosphorylated in vitro by Src and removed from the beads by exposure to SDS and 1% β-mercaptoethanol (see “Experimental Procedures”). An aliquot of the sample was directly analyzed by Western blotting with p120 mAb (lane 1) or GST linked to the SH2 domains of SHP-1 (lanes 2–7). **A** contain wild type p120–1A (lanes 1 and 2) or p120–1A with combinations of Tyr→Phe mutations at residues 96, 112, 228, 257, 280, 291, 296, and 302 (lane 3); residues 96, 112, and 228 (lane 4); residues 257, 280, 291, 296, and 302 (lane 5); residues 257 and 280 (lane 6); or residues 291, 296, and 302 (lane 7).

**Fig. 7.** Schematic showing location of Src-induced p120 tyrosine phosphorylation sites in p120. The locations of unphosphorylated (filled circles) and phosphorylated (open circles on stems) tyrosines are indicated relative to the overall domain structure of p120. All of the major Src phosphorylation sites reside in the amino terminus. Six of the cluster in a short region amino-terminal to the first Armadillo repeat (shaded), which we refer to as the phosphorylation domain (batched). RNA splicing leads to alternative usage of four different ATG start sites (designated by arrows 1–4). Isoforms 1 and 3 are the most abundant forms, whereas isoforms 2 and 4 are rarely observed.
rule out some contribution from the carboxyl-terminal SH2 domain. However, assaying several combinations of Tyr→Phe p120 mutants suggested that the strength of the interaction best correlated with the number of p120 phosphotyrosine residues left intact. Thus, although p120 tyrosine phosphorylation was clearly prerequisite for SHP-1 binding, we were unable to identify particular sites that specifically mediated the SHP-1 interaction. The epidermal growth factor receptor phosphorylation domain behaves similarly with regard to Shc, which binds to each of the epidermal growth factor receptor autophosphorylation sites with some degree of preference but without absolute specificity for a single site (42).

Because tyrosine phosphorylation of p120 was originally reported to correlate with Src-induced cell transformation, we overexpressed our p120–8F mutant in Src transformed NIH3T3 or MDCK cells to screen for any obvious dominant negative effects. Despite the inability of the p120–8F protein to be phosphorylated by Src, expression of this mutant did not ported to correlate with Src-induced cell transformation, we identify particular sites that specifically mediated the SHP-1 events, thereby directly implicating the site in p120 function. Moreover, it should now be possible to efficiently screen for putative p120 phosphotyrosine-dependent binding partners and to generate p120 tyrosine phospho-specific monoclonal antibodies, developments that will greatly facilitate the identification of upstream and downstream events associated with p120 tyrosine phosphorylation.

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Identification of Src Phosphorylation Sites in the Catenin p120<sup>ctn</sup>
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