The Src family tyrosine kinases and their substrates are involved in cell-cell and cell-matrix interactions. We found that in PC12h cells, an increase of cell density enhanced the tyrosine phosphorylation levels of several intracellular proteins including p130Cas. Because it is a possible substrate for Src family kinases, we measured pp60v-src activity and found that it was higher in high density cultures than in low density cultures. This phenomenon was also observed in PC12 (the parental cell line of the PC12h subclone), Balb/c 3T3, Swiss 3T3, and Hela cells. One of the possible mechanisms regulating the kinase activity of pp60v-src is the phosphorylation and dephosphorylation of its negative regulatory site located at its C terminus. However, the tyrosine phosphorylation level of the regulatory site did not change depending on cell density. Subcellular fractionation showed that in high density culture, pp60v-src was translocated from detergent-soluble to detergent-insoluble fractions. These results suggest that cell-cell interaction might induce the activation of pp60v-src without changing its tyrosine phosphorylation levels.

The Src family kinases are nonreceptor tyrosine kinases expressed in a wide variety of cells including mature nondividing neurons. They have been implicated not only in proliferation but also in cell adhesion, cytoskeletal organization, and differentiation (1, 2). The N-terminal half contains the SH2 and SH3 domains that could interact with certain proteins having phosphorylated tyrosine residues (3, 4) and polyproline-like helices (5, 6), respectively. The C-terminal half is comprised of the catalytic domain and a noncatalytic tail (7). The intramolecular interactions of these domains are thought to be important in regulation of these kinases.

One of the possible functions of Src family kinases is to regulate cell-cell and cell-matrix adhesions by phosphorylating proteins of the adhesion plaques. Overexpression of pp60v-src in fibroblasts elevates the tyrosine phosphorylation levels of proteins including paxillin, cortactin, and pp125FAK (8). All these proteins are localized at focal contacts where they form complexes with integrins and other proteins to anchor the actin fibers to the site of cell contact (9, 10). In v-src-transformed cells, pp60v-src is localized at the adhesion plaques, and the amount of actin stress fibers is greatly reduced (11). These findings indicate that regulation of Src family kinases is essential to integrity of the actin cytoskeleton (1).

p130Cas is another protein that is highly tyrosine-phosphorylated in v-src-transformed cells (8, 12). It binds to the SH3 and SH2 domains of pp60v-src and the SH2 domain of c-Crk in a tyrosine phosphorylation-dependent manner (13–15). The binding site of p130Cas for the SH3 domain of pp60v-src has been identified as a C-terminal proline-rich sequence (13). The distribution of p130Cas is not yet known, but its tyrosine phosphorylation is stimulated when cells are attached on fibronectin-coated dishes, suggesting that it is involved in the intracellular signaling pathway of integrins (10, 16).

Activities of Src family kinases are regulated by several different mechanisms. Under normal growth conditions, they are suppressed by tyrosine phosphorylation of the negative regulatory sites located at their C-terminal noncatalytic tails (1). These sites are phosphorylated by CSK, another tyrosine kinase (17, 18), and dephosphorylated by some tyrosine phosphatases. In cells lacking CSK, the activities of Src family kinases are elevated, and paxillin and cortactin are highly tyrosine-phosphorylated (19). These cells are able to undergo normal proliferation but have disorders in focal contact formation and localization of some focal adhesion-associated proteins including paxillin, supporting the importance of Src family kinases in cell adhesion.

Src family kinases are known to be activated in response to growth factors (20, 21), UV irradiation (22), and transition to the M phase in the cell cycle (23–25). However, it is not fully understood when pp60v-src and related proto-oncogene products are activated in normal cells. During the course of the study on nerve growth factor-induced tyrosine phosphorylation in PC12h cells (26), we have noticed that tyrosine phosphorylation levels of some proteins were changed depending on cell density. This prompted us to study the effect of cell density on tyrosine phosphorylation of intracellular proteins and on activities of tyrosine kinases. In the present report, we show that activities of Src family kinases are elevated in high density culture and discuss the mechanism underlying it.

**EXPERIMENTAL PROCEDURES**

Materials—PC12h cells were kindly provided by Dr. H. Hatanaka of our institute. PC12, Balb/c 3T3, Swiss 3T3, and Hela cells were provided by the Japanese Collection of Research Biologicals (Health Science Research Resources Bank) Cell Bank (Japan). Horseradish peroxidase-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were from Zymed. [γ-32P]ATP, protein G-Sepharose FF, and protein A-Sepharose FF were from Amersham Corp. [3P]Orthophosphate was from ICN. Anti-v-Src monoclonal antibody (clone 327) was from Oncogene Science, anti-phosphotyrosine (clone 4G10) was from Upstate Biotechnology, Inc., and anti-phosphotyrosine (clone PY-20) and anti-FAK antibody were from Transduction Laboratories.
CSK antisera were prepared by immunizing rabbits with GST-Cas2 (15) and GST-CSK, respectively.

Cell Culture and Immunoprecipitation—PC12 and PC12h cells were maintained in DMEM containing 5% horse serum and 5% calf serum as described previously (18). For experiments, the cells were plated on 100-mm culture dishes at different densities as indicated and cultured for 2 days. For immunoprecipitation, cells were lysed in 0.5 ml of TNE buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μM sodium molybdate, 10 μM aprotinin, and 10 μM leupeptin), and after protein determination the protein concentrations were adjusted. The lysates (500 μg of protein each) were incubated with primary antibody for 1 h at 4°C and then with protein G-Sepharose or protein A-Sepharose for 1 h at 4°C. The immune complex was washed three times with TNE buffer, resuspended in 20 μl of TNE buffer and 20 μl of 2 × SDS-PAGE sample buffer, and then boiled for 3 min. Proteins were determined by the method of Bradford (27) with bovine serum albumin as a standard. Balb/c 3T3, Swiss 3T3, and Hela cells were maintained in DMEM containing 10% calf serum and subcultured every 6–8 days.

SDS-PAGE and Immunoblotting—Proteins were separated by SDS-PAGE on a 8% polyacrylamide gel by the method of Laemmli and were electrophoretically transferred to a nitrocellulose membrane. The filter was then blocked in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) and incubated with primary antibody for 1 h. After washing with T-TBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. Blots were developed with the Renaissance chemiluminescence Western blotting detection kit (NEN Life Science Products).

In Vitro Kinase Assay of pp60 c-src—Cells were lysed in RIPA buffer (TNE buffer containing 0.5% sodium deoxycholate, 0.1% SDS), and pp60 c-src was immunoprecipitated with anti-v-Src antibody. The immune complexes were washed three times with TNE buffer and twice with kinase buffer (10 mM PIPES, pH 7.0, 10 mM MnCl2). In vitro kinase reactions were carried out in 40 μl of kinase buffer containing 10 μg of enolase and 10 μM γ-32P[ATP (10 μCi) at 30°C for 30 min and stopped by the addition of 40 μl of 2 × sample buffer. The samples were boiled and separated on a 8% gel, and then the gel was dried and exposed to an x-ray film for 1 h.

CNBr Cleavage of 32P-Labeled pp60 c-src—Cells were metabolically labeled with 1 μCi/ml of [32P]orthophosphate in phosphate-free DMEM for 16 h and lysed in RIPA buffer. pp60 c-src was immunoprecipitated and subjected to electrophoresis on a 8% SDS-polyacrylamide gel. After drying the gel, pp60 c-src was detected by autoradiography, cut out from the dried gel, and digested with CNBr (0.5 mM in 70% formic acid) for 1 h at room temperature. After digestion, the gel was washed with 10% methanol, dried up, and homogenized with elution buffer (10 mM NaHCO3, 1% β-mercaptoethanol, 0.1% SDS). The gel was then boiled for 3 min, shaken for 1 h, and centrifuged, and the supernatant was dried up. The samples were dissolved in SDS sample buffer without SDS, boiled for 3 min, and separated by Tris-Tricine-SDS-PAGE (28). The radioactive bands were detected by autoradiography.

RESULTS

In the course of the study on nerve growth factor-induced tyrosine phosphorylation in PC12h cells, we have noticed that the tyrosine phosphorylation levels of some intracellular proteins had been changed depending on cell density. To examine the density-dependent change in tyrosine phosphorylation, we prepared PC12h cell cultures of different cell densities. PC12h cells were plated at 5 × 104 for low density culture and 2 × 105 for high density culture. After 2 days, the cell densities were essentially equal in all samples as shown by immunoblotting of the same samples with anti-p130 Cas2 antibody, and measured its activity in high density culture was 2.5-fold higher than activity in a low density culture. The autophosphorylation activity was also increased to a similar extent, but the amount of pp60 c-src did not change in each condition as detected by

Fig. 1. Morphology of PC12h cells growing at different cell densities. PC12h cells were plated at 5 × 104 cells/cm2 for a low density culture and 2 × 105 cells/cm2 for a high density culture. After 2 days, phase contrast micrographs were taken, and the number of cells was counted. The cell densities were 1 × 104 cells/cm2 (A) and 4 × 105 cells/cm2 (B).

Because p130 Cas2 is a possible substrate for pp60 c-src, these results raised the possibility that the kinase activity of pp60 c-src could be changed depending on cell density. To test the possibility, we immunoprecipitated pp60 c-src and measured its activity with enolase as the substrate (Fig. 3A). It was found that the pp60 c-src activity in high density culture was 2.5-fold higher than activity in a low density culture. The autophosphorylation activity was also increased to a similar extent, but the amount of pp60 c-src did not change in each condition as detected by phosphorylated proteins of 120–130 kDa, including p130 Cas2 and p125 FAK. To investigate whether the tyrosine phosphorylation levels of p130 Cas2 were changed depending on cell density, p130 Cas2 was immunoprecipitated from these lysates with anti-p130 Cas2 antibody and then analyzed by immunoblotting with anti-phosphotyrosine antibody. In low density culture, p130 Cas2 exhibited a low level of tyrosine phosphorylation, whereas it was highly tyrosine-phosphorylated in high density culture (Fig. 2B). The amounts of p130 Cas2 protein were essentially equal in all samples as shown by immunoblotting of the same samples with anti-p130 Cas2 antibody. The changes in tyrosine phosphorylation of p130 Cas2 was also confirmed by immunoprecipitation with anti-phosphotyrosine antibody followed by Western blotting with anti-p130 Cas2 antibody (Fig. 2C). On the other hand, when the immunoprecipitates with anti-phosphotyrosine antibody were probed with anti-p125 FAK antibody, we could not detect any change in tyrosine phosphorylation level of p125 FAK (Fig. 2C).
Western blotting.

We next examined whether the total tyrosine phosphorylation level of pp60<sup>src</sup> was changed depending on cell density. In pp60<sup>src</sup> cells, there are two tyrosine phosphorylation sites: the autophosphorylation site and the negative regulatory site (corresponding to the Tyr<sup>416</sup> and Tyr<sup>527</sup> of chicken c-Src, respectively). In PC12h cells, however, tyrosine phosphorylation occurs predominantly on the negative regulatory site as shown later (see Fig. 5). Thus the total tyrosine phosphorylation level would reflect that of the negative regulatory site. As shown in Fig. 3B, there was no difference in the total tyrosine phosphorylation levels of pp60<sup>src</sup> between low and high density cultures, suggesting that the tyrosine phosphorylation level of the negative regulatory site did not change depending on cell density.

The negative regulatory site is phosphorylated primarily by CSK, but the only known mechanism that controls CSK is regulation of its expression levels. Western blotting with anti-CSK antibody showed that its expression level did not change depending on cell density (Fig. 3C). It is consistent with the result that the total tyrosine phosphorylation levels of pp60<sup>src</sup> did not change (Fig. 3B).

Next we investigated whether the density-dependent change in pp60<sup>src</sup> activity is specific to PC12h cells or not. PC12h is a subclone derived from PC12 cells (29). This subclone has no detectable difference in morphology and nerve growth factor-induced neurite extension but expresses higher tyrosine hydroxylase activity than the parental cells on stimulation with nerve growth factor. As shown in Fig. 4A, the parental cells also exhibited density-dependent increase in pp60<sup>src</sup> activity as is the case with PC12h subclone. We further investigated using three fibroblastic cell lines, Swiss 3T3, Balb/c 3T3, and Hela. These cells are immortal but not transformed, and their growth is restricted by contact inhibition. In all these cells, pp60<sup>src</sup> was also activated in high density cultures, whereas its expression levels were almost constant (Fig. 4B). Thus the phenomenon found in PC12h cells could be observed in many other cell lines.

The two tyrosine phosphorylation sites of pp60<sup>src</sup> could be distinguished by phosphopeptide mapping after CNBr digestion (30). Fig. 5A shows the phosphopeptide mapping of pp60<sup>src</sup> after in vitro labeling in the presence and the absence of GST-CSK. In the absence of GST-CSK, phosphorylation occurs exclusively on the 10-kDa fragment, which should contain the autophosphorylation site, whereas in the presence of GST-CSK, phosphorylation occurs on the 4-kDa fragment containing the negative regulatory site in addition to the 10-kDa fragment. This result confirms that phosphopeptide mapping can be used to distinguish the phosphorylation on Tyr<sup>416</sup> and Tyr<sup>527</sup>.

To identify the phosphorylation sites of pp60<sup>src</sup> in vivo, PC12h cells were metabolically labeled with [32P]orthophosphate, and 32P-labeled pp60<sup>src</sup> was immunoprecipitated with anti-v-Src antibody and subsequently digested with CNBr (Fig. 5B). The major phosphorylation site of pp60<sup>src</sup> was on the 31-kDa fragment containing the N-terminal serine phosphorylation.
FIG. 4. Density-dependent change in pp60<sup>c-src</sup> activity in various cell lines. A, lysates (500 μg of protein) from "original" PC12 cells growing at different cell densities (1 × 10<sup>5</sup> (lane 1), 2 × 10<sup>5</sup> (lane 2), 4 × 10<sup>5</sup> (lane 3), and 8 × 10<sup>5</sup> (lane 4) cells/cm<sup>2</sup>) were subjected to immunoprecipitation with anti-pp60<sup>c-src</sup> antibody. Then the immunoprecipitates were incubated with [γ-32P]ATP and enolase in the presence of 10 mM MnCl<sub>2</sub>. The labeled proteins were separated on two 8% SDS-polyacrylamide gels. One gel was then exposed to an x-ray film to determine the kinase activity, and the other was analyzed by Western blotting with anti-v-src antibody. The signals were quantified by the NIH Image software. Relative specific activity of each sample was calculated by dividing the kinase activity by the protein amount. B, lysates from Swiss 3T3, Balb/c 3T3, and Hela cells of different cell density (3 × 10<sup>4</sup> (lane 1), 6 × 10<sup>4</sup> (lane 2), 1 × 10<sup>5</sup> (lane 3), and 2 × 10<sup>5</sup> (lane 4) cells/cm<sup>2</sup>, respectively) were analyzed as described above.

DISCUSSION

In the present study, we first examined the tyrosine phosphorylation levels of cellular proteins in PC12h cells grown at different cell densities and found that the tyrosine phosphorylation levels of several proteins were elevated when the cell density became high. In low density cultures, each cell was separated from other cells, whereas in high density cultures, cells were confluent and made contact with each other. The changes in tyrosine phosphorylation might be due to the influence of the cell-cell interaction. In addition, similar changes were also observed in several other types of cells including PC12, Balb/c 3T3, Swiss 3T3, and Hela, indicating that this phenomenon is not unique to PC12h cells, rather commonly seen in a variety of transformed and nontransformed cells.

One of the proteins that were highly tyrosine-phosphorylated at high cell density was identified as p130<sup>Cas</sup>, a docking protein that can be associated with the SH2 domains of pp60<sup>c-src</sup> (13) and c-Crk (15). p130<sup>Cas</sup> was first found as a highly tyrosine-phosphorylated protein in v-src-transformed fibroblasts, and so it was thought to be one of the substrates of pp60<sup>c-src</sup> (8, 12). This was confirmed by the finding that tyrosine phosphorylation level of p130<sup>Cas</sup> was very low in c-src-deficient cells (34). Thus the increase of the tyrosine phosphorylation levels of p130<sup>Cas</sup> indicated that pp60<sup>c-src</sup> or related proteins were activated in high density cultures. PC12h cells have several Src family kinases including pp60<sup>c-src</sup>, pp59<sup>c-fyn</sup>, and pp52<sup>c-yes</sup>. In the present study, we showed that pp60<sup>c-src</sup> activity was activated in high density cultures. Src family kinases are known to be involved in a variety of cellular activities. Expressions of the activated forms of these kinases result in abnormal cell growth, disorder of the actin cytoskeleton, and incomplete cell adhesion. However, only a few reports showed changes in their activities under normal growth conditions (35). Thus the present results would be important in understanding their physiological function in normal growth conditions (35).
The strongest band was a 31-kDa band that contains the N-terminal serine phosphorylation site. It is also thought to be involved in regulation of pp60^{src} activity (1), but its phosphorylation levels were not different between low and high density cultures. Therefore, serine phosphorylation of its N terminus was also unlikely to be involved in the density-dependent activation. But we could not exclude the possibility that the serine phosphorylation site might be different in low and high density cultures because the 31-kDa fragment of CNBr digestion contains more than one serine and threonine residues. Further studies are needed to clarify the activation mechanisms.

In normal cells, pp60^{src} is usually solubilized in a buffer containing 1% Triton X-100, whereas in v-src-transformed cells, pp60^{src} was found in Triton-insoluble fractions that contain various cytoskeletal components (32, 33), suggesting that the activated form of the kinase is tightly associated with the cytoskeleton. In low density cultures, pp60^{src} was mostly found in Triton-soluble fractions. On the other hand, in high density cultures, about 10% of it was Triton-insoluble. In high density cultures, pp60^{src} might thus be activated and translocated to some cytoskeletal structures. It is not known what is associated with pp60^{src} to make it Triton-insoluble.

The present study suggested that Src family kinases were activated by cell-cell contacts. Consistent with this, a comparative study (37) showed that Src family kinases could be found in sponge, the first multicellular organisms, but not in unicellular organisms, implying that these may be involved in cell-cell interaction, recognition, and aggregation. It remains to be determined which molecule triggers the activation of Src family kinases. Recently, it was found that stimulation of integrin caused tyrosine phosphorylation of pp125^{FAT} (20) and pp130^{crk} (10, 16), suggesting that they are downstream of integrin signaling. However, the tyrosine phosphorylation levels of pp125^{FAT} did not change depending on cell density in contrast to pp130^{crk}. Some other pathways might thus be responsible for the tyrosine phosphorylation of pp130^{crk} in high density cultures. It has been reported that some cell adhesion molecules such as L1 and N-CAM require Src family kinases for their function (38, 39). These molecules might be stimulated in high density cultures and affected downstream signal transduction molecules including Src family kinases.

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Activation of pp60<sup>src</sup>