The protein tyrosine kinase expressed by the proto-oncogene c-fgr is phosphorylated and down-regulated in vitro by the c-Src kinase (CSK). CSK catalyzed phosphorylation affects Tyr-511 of c-Fgr, homologous to Tyr-527 of c-Src and it prevents the autophosphorylation normally occurring at c-Fgr Tyr-400, homologous to c-Src Tyr-416. Polylysine, histones H1 and H2A and other polycationic proteins on the other hand stimulate c-Fgr activity while promoting enhanced autophosphorylation of both Tyr-400 and Tyr-511. Once phosphorylated at Tyr-511 and down-regulated by CSK, c-Fgr is no more susceptible to polylsine stimulation. Previous autophosphorylation (at Tyr-400) reduces c-Fgr susceptibility to down-regulation by CSK, although Tyr-511 can be still phosphorylated by it. If a more exhaustive autophosphorylation (of both Tyr-400 and Tyr-511) is performed in the presence of polylsine, c-Fgr becomes totally insensitive to CSK down-regulation.

These data support the concept that down-regulation of c-Fgr by Tyr-511 phosphorylation is prevented if Tyr-400 is also phosphorylated and they are consistent with an outcompetition of phospho-Tyr-511 from the Src homology 2 domain by phospho-Tyr-400, which, in c-Fgr, is surrounded by an amino acid sequence divergent from that of the other Src-related protein tyrosine kinases.

Src-related protein tyrosine kinases make up a family of nonreceptor enzymes termed after the product of the proto-oncogene c-src, the cellular counterpart of v-src, whose product, v-Src, was the first protein tyrosine kinase to be detected (Collet and Erikson, 1978; Hunter and Sefton, 1980). Nowadays, nine members of the src family are known, namely c-Src, c-Yes, Lyn, Fyn, Lck, Hck, c-Fgr, Blk, and Yrk (Cooper, 1990; Cooper and Howell, 1993).

All of these display a remarkable homology and share a common structure, consisting of a N-terminal, poorly conserved segment, followed by an SH3 and an SH2 domains, both upstream from the catalytic domain. This includes an highly conserved autophosphorylation site (Tyr-416 in c-Src), whose phosphorylation is generally believed to correlate with up-regulation of catalytic activity (Cooper and Howell, 1993). In the cellular members of the Src family, but not in the viral ones, the catalytic domain is followed by a short regulatory C-terminal tail including a tyrosyl residue (Tyr-527 in c-Src), whose phosphorylation in c-Src and Lck was unambiguously shown to inhibit the catalytic activity of these kinases (Marth et al., 1988; Cooper and King, 1986; Courtneidge, 1985; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987; Cartwright et al., 1987). A protein tyrosine kinase has been isolated (Okada and Nakagawa, 1988) and cloned (Nada et al., 1991) which is capable to phosphorylate this Tyr-527 residue and down-regulate c-Src (Okada and Nakagawa, 1989). The mechanism by which such a negative regulation occurs involves an interaction between the phosphorylated Tyr-527 and the SH2 domain of the kinase itself (Cantley et al., 1991; Superti-Furga et al., 1993; Cooper and Howell, 1993) giving rise to an inactive conformation of the enzyme. Besides c-Src, other members of the Src family, namely Fyn, Lyn (Okada et al., 1991) and Lck (Bergmann et al., 1992) have been shown to undergo phosphorylation and down-regulation by CSK. Conversely, nothing was known about the regulation of c-Fgr, a member of the Src family expressed in normal and malignant B lymphocytes infected with Epstein-Barr virus (Cheah et al., 1986), as well as in normal peripheral blood monocytes and granulocytes and in alveolar and splenic macrophages (Ley et al., 1989).

The biochemical properties of c-Fgr, whose autophosphorylation site shares an only limited homology with that of the other members of the family (Katamine et al., 1988), have hardly been investigated. We have recently shown that a protein tyrosine kinase previously isolated from rat spleen and conventionally termed tyrosine protein kinase-III (Brunati and Pinna, 1988), once submitted to MonoQ fast protein liquid chromatograhy gives rise to a predominant peak of activity (tyrosine protein kinase-III Q1) due to an enzyme that was identified as c-Fgr, based on both immunological and partial sequencing evidences (Brunati et al., 1993). Here we report a biochemical study on rat spleen tyrosine protein kinase-III Q1, henceforth termed c-Fgr, and in particular on its regulation by CSK and by polycationic proteins that have been shown to stimulate the activity of other protein tyrosine kinases (Gatica et al., 1987; Fujita-Yamaguchi et al., 1989; Abdel-Ghany et al., 1990; Donella-Deana et al., 1992).

**EXPERIMENTAL PROCEDURES**

Polylysine (average molecular mass of 46 kDa) was purchased from Sigma, [(γ-32P)ATP from Amersham Corp. The peptide EDDNAYTA was nylbenzoyl 5'-adenosine; MS/MS, two mass scanning devices linked together for fragmentation studies to determine peptide sequences.
synthesized by a manual synthesizer (model Biolinx 4175, LKB), as detailed by Marin et al. (1991); the peptides KDDDEYTPA and EPQQYQPA were synthesized by an automated synthesizer from Applied Biosystem (model 431-A) (Brunati et al., 1992).

c-Fgr tyrosine protein kinase was purified from the particulate fraction of rat spleen, according to the procedure elsewhere reported (Brunati et al., 1993). Its Tyr(P) content was negligible as judged by lack of reactivity with anti-Tyr(P) antibodies (monoclonal Py20, from ICN Biochemical). CSK was partially purified from rat spleen cytosol, as described by Brunati et al. (1992).

c-Fgr inactivation by FSBA was performed by 1 h incubation at 30 °C with 1 mM FSBA in the presence of 5 mM MgCl₂; the residual FSBA was quenched by addition of 5 mM 2-mercaptoethanol (Okada and Nakagawa, 1989).

For the evaluation of 32P incorporated, the reactions were stopped by addition of 2% SDS, and the samples were subjected to 11% SDS-PAGE, followed by autoradiography.

For CNBr digestion, the phosphorylation reactions were stopped with formic acid (70% final concentration) and the cleavage was performed essentially according to Luo et al. (1991). Briefly, CNBr was added to a final concentration of 50 mg/ml; the reaction was allowed to continue for 1 h at room temperature in the dark, and stopped by dilution to 1:10 and lyophilization. The samples, resuspended in gel electrophoresis buffer containing 2% SDS, were subjected to 20% SDS-PAGE (Laemmli, 1970), followed by autoradiography.

Phosphorylation of the peptides was performed in the same medium described for autophosphorylation reaction, in the presence of the substrate peptide at the concentration indicated in the legend of the figures. The reaction was stopped by adding acetic acid (final concentration 30% v/v), and 32P incorporated into the peptide was evaluated by combining ion exchange and isobutanol/toluene extraction, as detailed previously (Cola et al., 1989).

Kₚ and Vₘₚ values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis-Menten equation. The values reported are the means of at least three separated experiments; the S.E. value was less than 15%.

Peptide microsequencing was performed as follows: autophosphorylated c-Fgr was reduced in 20 µl of a buffer containing 2 mM dithiothreitol, 8 M urea, 0.1 M Tris, pH 8.5, for 15 min at 37 °C. Iodoacetic acid was added to a final concentration of 3 mM and the pH adjusted to 8.5 with Tris-base, and incubated for 2 h at 37 °C before quenching the reaction with dithiothreitol to a final concentration of 5 mM. The solution was diluted 5-fold and trypsin added (1:100, w/w) and the digestion carried out for 12 h at 37 °C. The digestion was terminated by acidification to pH 2.0. Peptides were separated on a Brownlee (Applied Biosystems, Foster City, CA) C-18, 300 A, 1 x 100-mm reversed phase column. Fractions were collected manually. Fractions containing radioactivity were analyzed by Edman degradation on an ABI 475 protein sequenator with on line phenylthiohydantoin detection and also by electrospray ionization mass spectrometer. Chymotryptic subdigestion was carried out (1:25, w/w) and the peptides were separated as described above.

Sucrose density gradient analysis was performed with a linear gradient (5 ml) of sucrose 5–20%, w/w in 50 mM Hepes, pH 7.0, containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. Unphosphorylated or previously autophosphorylated c-Fgr (150 ng) was loaded on top of the gradient and centrifuged at 48,000 rpm in a Beckman TSW 60 rotor for 15 h at 4 °C. 0.2-ml fractions were collected and monitored for enzymatic activity.

RESULTS

Phosphorylation of c-Fgr under Different Conditions—The radiolabeled CNBr fragments of c-Fgr phosphorylated by [γ-32P]ATP under various conditions were isolated by SDS-PAGE and autoradiography (Fig. 1A). Autophosphorylation under basal conditions gives rise to two radiolabeled bands of apparent 20,000 and 10,000 Mₐ (lane 1). The intensity of both these bands decreased while a new radiolabeled band of 5 kDa became prominent if the phosphorylation was performed in the presence of CSK (lane 2). The 5-kDa radiolabeled band is even more evident if c-Fgr is autophosphorylated in the presence of polylysine; in this case, however, the other two bands, far from disappearing, become more intense (lane 3). Increased radiolabeling of the 20- and 10-kDa bands and appearance of the 5-kDa band were also observed by replacing polylysine with histones H1 and H2A and protamine, but not with spermine (not shown). The stimulatory effect of polylysine on c-Fgr autophosphorylation almost disappears if c-Fgr is previously incubated with CSK; in this case only the 5-kDa band is evident, while the 20- and 10-kDa bands are faint as when CSK alone is added (lane 4). Previous inactivation of c-Fgr with FSBA does not prevent its phosphorylation by CSK at the site(s) included in the 5-kDa band (lane 7), while, as expected, autophosphorylation is prevented either in the absence or in the presence of polylysine (lanes 6 and 8, respectively). Polylysine has no effect on CSK catalyzed phosphorylation of FSBA-inactivated c-Fgr (lane 9), consistent with the concept that CSK itself is insensitive to polylysine.

FIG. 1. CNBr 32P-peptide maps of c-Fgr phosphorylated under different conditions. A, c-Fgr (150 ng) either native (lanes 1–4) or previously inactivated by FSBA (lanes 5–9) was incubated at 30 °C for 20 min in the autophosphorylation medium (described under "Experimental Procedures") either alone (lanes 1 and 6) or with the following additions: CSK, 300 ng (lanes 2 and 7); polylysine, 10 µg/ml (lanes 3 and 8); CSK plus polylysine (lane 9). In the experiment of lane 4, polylysine (10 µg/ml) was added after 50 min of incubation with CSK, and the incubation was continued for 10 min more. In the experiment of lane 5, CSK alone was incubated at 30 °C for 20 min in the same autophosphorylation medium as for c-Fgr. B, c-Fgr was incubated as in Panel A, either alone for 20 min (lane 12), or with CSK, for 5 min (lane 10). In the experiment of lane 11 CSK was added after 15 min of preincubation, and the incubation was continued for 5 min more. At the end of incubation, each sample was digested with CNBr and the radiolabeled fragments were resolved by 20% SDS-PAGE and evaluated by autoradiography. The positions of molecular mass markers (in kDa) are indicated on the right.
These data show that c-Fgr is phosphorylated by CSK at a site which is not appreciably affected by autophosphorylation under basal conditions, but which is autophosphorylated if c-Fgr is incubated in the presence of polylysine. It should also be noted that previous autophosphorylation of c-Fgr does not affect its phosphorylation by CSK, which is still capable to induce the appearance of the 5-kDa radiolabeled band (Fig. 1B, compare lanes 10 and 11). The resulting pattern is reminiscent of that observed upon incubation of c-Fgr alone with polylysine, in that all the three CNBr radiolabeled peptides are evident. Attempts have been made to calculate the kinetic constants for the phosphorylation of c-Fgr by CSK, using FSBA-inactivated c-Fgr as phosphorylatable substrate. A linear increase of phosphorylation however was observed up to 0.6 μM c-Fgr (i.e., the highest concentration allowed by our experimental conditions) which hindered the evaluation of either K_m or V_max.

Results similar to those obtained upon CNBr digestion were got if radiolabeled c-Fgr was digested with trypsin, and the fragments were isolated by reversed phase HPLC on a C-18 column (not shown). In this case, however, only one (instead of two) and two (instead of three) radiolabeled peptides were isolated from c-Fgr autophosphorylated under basal conditions and in the presence of polylysine, respectively (see below). These observations in conjunction with the quantitative analysis of radiolabeled peptides detected by either the CNBr/SDS-PAGE or the trypsin/HPLC procedure (Table I) support the view that the radiolabeled 20-kDa phosphopeptide detected on SDS-PAGE is accounted for by the same site as in the 5-kDa peptide, either due to incomplete cleavage, or to SDS-resistant association with other fragments. This conclusion would be also consistent with the observation that the radiolabeling of the 20-kDa and the 10-kDa peptides is always varying in parallel (see e.g. Fig. 1) and with the detection of only two phosphorylated tyrosyl residues by mass spectrometry analysis of c-Fgr phosphorylated in the presence of polylysine (see below) instead of the three suggested by the SDS-PAGE pattern of Fig. 1.

Identification of the Phosphoacceptor Sites—By analogy with other protein tyrosine kinases of the Src family, it was expected that c-Fgr autophosphorylation under basal conditions and its phosphorylation by CSK would take place at Tyr-400 (homologous to c-Src Tyr-416) and Tyr-511 (homologous to c-Src Tyr-500), respectively. The molecular masses of CNBr peptides of 10 and 5 kDa, respectively, were consistent with such an identification. To identify unambiguously the residues phosphorylated, microsequencing and mass spectrometry analysis were performed on a sample of c-Fgr phosphorylated in the presence of polylysine. These conditions were chosen because, (i) they promote the highest incorporation of phosphate, in all the CNBr peptides (see Fig. 1); (ii) it was important to ascertain whether the polylysine driven autophosphorylation of the 5-kDa fragment specifically affected by CSK actually occurred at the CSK site (i.e. Tyr-511) or at other tyrosyl residue(s) present in that fragment. Upon tryptic digestion of the carboxymethylated material followed by C-18 reversed phase HPLC, two radioactive peaks were resolved, termed A and B (Fig. 2). Peptide A was loaded on the mass spectrometer and showed a weight of 1700. This fragmented well in MS/MS and gave the following sequence: LIVDDEYPQYSPQGTK (see Fig. 3), which corresponds to the expected autophosphorylation site of c-Fgr, homologous to Tyr-416 of c-Src.

The second tryptic peak (B) gave molecular weight of 3313 on the mass spectrometer. An aliquot of it was subjected to N-terminal sequencing revealing the peptide to be the C terminus of the protein. Since there are three tyrosines there that could be phosphorylated, the peptide was subdigested with chymotrypsin. A single radioactive peptide could be isolated which had a mass of 1241.8 on the mass spectrometer (not shown). Upon sequencing on the Edman sequencer, the results of Table II were found. They unambiguously show that the phosphopeptide is composed by the last ten amino acids of c-Fgr, including a single tyrosine at the fourth position. The failure to detect tyrosine at the fourth cycle is therefore quite consistent with its being phosphorylated. The presence of a phosphotyrosyl residue at the fourth position is confirmed by the calculated mass of the peptide sequenced (1161.8 being 80 mass units lower than that observed). This suggests the presence of a phospho group which fits with the knowledge that the peptide contains radiolabeled phosphotyrosine. It can be concluded therefore that the sequence around Tyr(P) in the second radiolabeled tryptic peptide is (E)PQYSPQGDQT, and the phosphorylated residue is Tyr-511.

In summary the sequencing data show that in the presence of polylysine c-Fgr undergoes autophosphorylation at both its canonical autophosphorylation site (homologous of c-Src Tyr-416) and the CSK phosphorylation site (homologous of c-Src Tyr-502). Endoproteinase Asp-N and CNBr digests of c-Fgr au-

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TABLE I

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Peptides</th>
<th>Radioactivity incorporated</th>
<th>c-Fgr</th>
<th>c-Fgr+polylysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNBr/PAGE</td>
<td>20-kDa</td>
<td>45</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-kDa</td>
<td>55</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-kDa</td>
<td>&lt;1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Trp ysin/HPLC</td>
<td>A</td>
<td>100</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>&lt;1</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

---

FIG. 2. C-18 reversed phase HPLC elution profile of tryptic phosphopeptides of c-Fgr autophosphorylated in the presence of polylysine. The autophosphorylated protein was reduced and carboxymethylated before digestion with trypsin. Peptides were separated on C-18, 300 A, 1 mm x 10-cm reversed phase columns using an ABI 140 A microbore system with a 1000S diode array detector. Peaks were collected manually and 1/20 of each was removed for liquid scintillation counting. Two peaks of radioactivity, labelled with arrows as A and B, were found. The yield of recovery of radioactivity compared to the intact carboxymethylated material prior to digestion was: A, 45%; B, 28%. The remaining radioactivity, eluted with the flow through of the column, was accounted for by inorganic phosphate, noncovalently bound to the protein.
**Regulation of c-Fgr Tyrosine Protein Kinase**

**Fig. 3. MS/MS analysis of radioactively labeled phosphopeptides obtained upon CNBr digestion of phosphorylated c-Fgr.**

The phosphopeptides obtained upon CNBr digestion of phosphorylated c-Fgr were isolated from the peptide fractions by HPLC and analyzed by tandem MS/MS. The spectra were recorded by selecting the parent ion (34 mass units wide at peak half height) in centroid mode. The b ions and y ions are shown at the bottom. Ions found in the daughter spectrum are indicated in bold face.

**Table II**

Automated Edman sequencing of a tryptic radioactive fragment of c-Fgr phosphorylated in the presence of polylysine (10 µg/ml).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Residue</th>
<th>pmol</th>
<th>Last 10 residues of rat c-Fgr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td>2</td>
<td>Pro</td>
<td>9.9</td>
<td>Pro</td>
</tr>
<tr>
<td>3</td>
<td>Glu</td>
<td>6.1</td>
<td>Glu</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td></td>
<td>Tyr</td>
</tr>
<tr>
<td>5</td>
<td>Glu</td>
<td>5.2</td>
<td>Glu</td>
</tr>
<tr>
<td>6</td>
<td>Pro</td>
<td>4.3</td>
<td>Pro</td>
</tr>
<tr>
<td>7</td>
<td>Gly</td>
<td>4.4</td>
<td>Gly</td>
</tr>
<tr>
<td>8</td>
<td>Asp</td>
<td>4.2</td>
<td>Asp</td>
</tr>
<tr>
<td>9</td>
<td>Glu</td>
<td>3.2</td>
<td>Glu</td>
</tr>
<tr>
<td>10</td>
<td>Thr</td>
<td>1.2</td>
<td>Thr</td>
</tr>
</tbody>
</table>

*Machine failure.

Consistent with a fully phosphorylated tyrosine residue which fails to be detected by Edman degradation (Turck et al., 1981).

**Table III**

Correlation between phosphorylation state and catalytic activity of c-Fgr.

<table>
<thead>
<tr>
<th>c-Fgr activity</th>
<th>Phosphorylation degree</th>
<th>Tyro-400</th>
<th>Tyro-511</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>pmol/min/µg</td>
<td>32.6</td>
<td>4.0</td>
</tr>
<tr>
<td>+ CSK</td>
<td>13.76</td>
<td>19.4</td>
<td>22.9</td>
</tr>
<tr>
<td>+ Polylysine</td>
<td>66.22</td>
<td>95.2</td>
<td>58.1</td>
</tr>
<tr>
<td>+ CSK/polylysine</td>
<td>31.00</td>
<td>51.7</td>
<td>39.7</td>
</tr>
</tbody>
</table>

**Fig. 4.** The dose dependent inhibitory effect of CSK decreases if c-Fgr is phosphorylated at Tyr-511 as well. Similar data were obtained with other polycationic proteins, namely histones H1 and H2A and protamines. The polybasic peptide CVVKILKPVKKKKKREIKILE affecting casein kinase 2 activity similarly to polylysine (Särno et al., 1993) was conversely ineffective up to 50 µM concentration (not shown). Consequently, in the presence of polybasic proteins the catalytic activity of c-Fgr is doubled despite its phosphorylation at Tyro-511 is even higher than that induced by CSK. The simultaneous addition of CSK and polylysine roughly neutralize each other: the catalytic activity is similar to that observed in the absence of any effector, while the phosphorylation state of Tyr-400 and Tyr-511 is intermediate between the values observed with either CSK or polylysine.

A more accurate analysis of the possible reciprocal influences of CSK and polylysine effects was performed by experiments in which these effectors were sequentially added. As shown in Fig. 4, the dose dependent inhibitory effect of CSK decreases if c-Fgr is phosphorylated at Tyr-511 as well. Similar data were obtained with other polycationic proteins, namely histones H1 and H2A and protamines. The polybasic peptide CVVKILKPVKKKKKREIKILE affecting casein kinase 2 activity similarly to polylysine (Särno et al., 1993) was conversely ineffective up to 50 µM concentration (not shown). Consequently, in the presence of polybasic proteins the catalytic activity of c-Fgr is doubled despite its phosphorylation at Tyr-511 is even higher than that induced by CSK. The simultaneous addition of CSK and polylysine roughly neutralize each other: the catalytic activity is similar to that observed in the absence of any effector, while the phosphorylation state of Tyr-400 and Tyr-511 is intermediate between the values observed with either CSK or polylysine.

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is preincubated with ATP-Mg and totally disappears if such a preincubation is performed in the presence of polylysine. On the other hand polylysine proved incapable of rescuing the activity of c-Fgr previously inactivated by CSK, as shown by the experiment of Fig. 5. The concept that c-Fgr is rendered insensitive to polylysine by previous phosphorylation by CSK is corroborated by the experiment in Fig. 6, where the stimulatory effect of increasing polylysine concentrations is tested on c-Fgr either preincubated or not with CSK. Altogether these data support the concept that, on one hand the inactive conformation of c-Fgr phosphorylated at its C-terminal tyrosine by CSK cannot be unlocked by polylysine, while, on the other, once phosphorylated at Tyr-400, c-Fgr is no more susceptible to down-regulation by CSK phosphorylation. Consistent with this latter concept is the non linear shape of the inactivation curve of c-Fgr by CSK (Fig. 4), suggestive that a number of c-Fgr molecules (presumably those which underwent autophosphorylation at Tyr-400) are refractory to inactivation. It has to be assumed therefore that in the presence of polylysine the phosphorylation of Tyr-400 precedes that of Tyr-511, in order to account for the finding that polylysine induces an overall stimulation of c-Fgr tyrosine protein kinase activity despite it also promotes a nearly stoichiometric phosphorylation of the down regulatory Tyr-511 site (see Table III). A sequential phosphorylation of Tyr-400 followed by Tyr-511 would be also consistent with the substrate specificity of c-Fgr, which, even in the presence of polylysine, by far prefers the peptide KDDEYNPA, reproducing the sequence around Tyr-400, over the peptide EPQQYPA, reproducing the sequence around Tyr-511 (Table IV). Such a preference is mostly due to the K_m value which is one order of magnitude lower with KDDEYNPA, and which is expected to play a more crucial role than V_max in the autophosphorylation of Src family protein tyrosine kinases, since this occurs through an inter-, rather than intramolecular mechanism (Cooper and MacAuley, 1988). It would be expected therefore that Tyr-400 is more readily phosphorylated than Tyr-511 upon polylysine driven autophosphorylation of c-Fgr. Clearly however polylysine also promotes the subsequent phosphophorylation of Tyr-511, which is almost undetectable upon autophosphorylation under basal conditions. This point is also outlined by the finding, shown in Fig. 7, that autophosphorylation in the presence of polylysine generates doubly phosphorylated c-Fgr, as judged by the upshift of the radiolabeled band on SDS-PAGE. Such an upshift is not evident either if c-Fgr is autophosphorylated in the absence of polylysine, or if it is phosphorylated by CSK (Fig. 7).

**DISCUSSION**

This study focusing on the regulation of the protein tyrosine kinase expressed by the proto-oncogene c-fgr, provides the following information. 1) Like other members of the Src family, namely c-Src (Okada and Nakagawa, 1989), Lck (Bergmann et
Regulation of c-Fgr Tyrosine Protein Kinase

Polylysine (μg/ml)  0  0.3  1  0
CSK        -   -   -   +

Fig. 7. Upshift of the c-Fgr band on SDS-PAGE upon autophosphorylation in the presence of polylysine. c-Fgr was autophosphorylated in the absence (lane 1), in the presence (lanes 2 and 3) of polylysine, or phosphorylated by CSK (lane 4). The radioactive protein was run on 11% SDS-PAGE, and detected by autoradiography. The positions of the molecular mass markers (in kDa) are shown on the right.

al., 1992), Lyn and Fyn (Okada et al., 1991), also c-Fgr is phosphorylated and down regulated by CSK. Such a phosphorylation occurs at Tyr-511 (homologous to c-Src Tyr-527) and correlates with both a decrease of catalytic activity toward peptide substrates and inhibition of autophosphorylation. 2) Autophosphorylation of c-Fgr under basal conditions involves Tyr-400 (homologous of c-Src Tyr-416) but not, to any appreciable extent, Tyr-511. Both Tyr-511 and Tyr-400, however, incorporate phosphate if autophosphorylation is performed in the presence of polycationic peptides, such as polylysine, histones H1 and protamines. Such a double phosphorylation induced by polylysine gives rise to an upshifted form of c-Fgr on SDS-PAGE and correlates with a stimulation of catalytic activity instead of a down-regulation, although the extent of Tyr-511 phosphorylation exceeds that promoted by CSK. 3) Once phosphorylated and down-regulated by CSK, c-Fgr becomes insensitive to polylysine stimulation in terms of both catalytic and Tyr-400 autophosphorylation activity. 4) The responsiveness to CSK down-regulation is reduced by previous autophosphorylation (at Tyr-400) although Tyr-511 phosphorylation by CSK still occurs. Insensitivity to CSK is complete if c-Fgr is preincubated with ATP in the presence of polylysine, promoting a nearly stoichiometric autophosphorylation of both Tyr-400 and Tyr-511.

From these data it can be concluded that while c-Fgr, like several other Src related protein tyrosine kinases, can be down-regulated by CSK through the phosphorylation of its C-terminal Tyr-511, such a phosphorylation looses its negative effect under circumstances favoring the autophosphorylation of Tyr-400, such as preincubation with ATP either under basal conditions, or, even more, in the presence of polylysine, which enhances c-Fgr autophosphorylation. Considering that down-regulation of the Src protein tyrosine kinases by CSK has been shown to occur through interaction between their C-terminal phosphotyrosyl residue (phosphorylated by CSK) and their SH2 domain, giving rise to a "locked" and inactive conformation of the kinase (Cantley et al., 1991; Superti-Furga et al., 1993; Cooper and Howell, 1993), the most plausible mechanism to explain our data is a competition between phosphorylated Tyr-400 and Tyr-511 for the same SH2 domain, as schematically shown in Fig. 8. According to this scheme, while the interaction of the SH2 domain with Tyr-511 would inactivate c-Fgr (as shown for other Src-related kinases), its interaction with Tyr-400 would not be detrimental or even might increase the catalytic activity, as suggested by the parallelism between hyperautophosphorylation of Tyr-400 and enhanced catalytic activity observed in the presence of polylysine. A successful outcompetition of the C-terminal phosphotyrosine by autophosphorylated Tyr-400 as proposed here would be disputable in the case of all of the other Src-related protein tyrosine kinases, due to the different sequences downstream from the two phosphotyrosines, the one of the C-terminal tyrosine exhibiting definitely higher affinity for the Src SH2 domain (Songyang et al., 1993). Such an outcompetition, however, is quite plausible in the case of c-Fgr, whose autophosphorylation site (Tyr-400) is unique among the Src kinases, both upstream (suggesting a different consensus for phosphorylation) and downstream, where it is reminiscent of the C-terminal Tyr-511 (Y,,,NPQ versus Y,,,QPG). As illustrated in Fig. 8, it has to be assumed that the interaction of the SH2 domain with either Tyr-400 or Tyr-511 gives rise to different conformations, since the latter inactivates while the former either is ineffective or even stimulates activity. Considering that autophosphorylation of Src kinases occurs through inter-rather than intramolecular mechanism, it is tempting to speculate that the active form of c-Fgr with phospho-Tyr400/SH2 interaction could be a dimer (A in Fig. 8). This could not be validated, however, by sucrose gradient ultracentrifugation experiments, showing that the apparent molecular mass of c-Fgr autophosphorylated in the presence of polylysine (55 kDa) is the one expected for the monomer and its mobility coincides with that of non autophosphorylated c-Fgr (data not shown). Consequently the model B of Fig. 8 is likely to be more correct, although it is also possible that the dimeric form dissociates under ultracentrifugation conditions.

The stimulatory effect of polylysine and other polycationic proteins on c-Fgr activity deserves some comments. These effectors play multiple roles since, besides stimulating catalytic activity, they also dramatically increase autophosphorylation of c-Fgr and they alter its specificity, since they trigger the autophosphorylation of Tyr-511 as well, which is unaffected under basal conditions.

While it is not clear whether the increased autophosphorylation of c-Fgr in the presence of polylysine is the cause or the consequence of its increased activity, it is worthy to note that it leads to an active doubly phosphorylated form of c-Fgr. Assuming that such a mechanism operates also in vivo (e.g. in the presence of polybasic peptides, like histones, whose effect is
mimicked in vitro by polylysine) it would give rise to an active form of c-Fgr, susceptible to inactivation by a protein tyrosine phosphatase(s) capable to specifically dephosphorylate Tyr-400 without affecting Tyr-511. By this hypothetical mechanism c-Fgr might undergo down regulation independently of CSK or any other kinase capable to phosphorylate its C-terminal domain. A similar mechanism could account for the observed down-regulation of Srco protein tyrosine kinases in the absence of CSK activity (MacAuley et al., 1993).

REFERENCES