Direct Binding of C-terminal Region of p130Cas to SH2 and SH3 Domains of Src Kinase

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Abstract

p130Cas is a major tyrosine-phosphorylated protein that tightly binds v-Crk in v-crk-transformed cells and v-Src in v-src-transformed cells. The "substrate domain" of p130Cas contains 15 possible Src homology (SH) 2-binding motifs, most of which conform to the binding motif for the Crk SH2 domain. Another region near its C terminus contains possible binding motifs for the Src SH2 domain and proline-rich sequences that are candidates for SH3-binding sites.

Using GST fusion proteins, we revealed that both SH2 and SH3 domains of Src bind p130Cas, whereas v-Crk binds p130Cas through its SH2 domain. We located the binding site of p130Cas for the Src SH3 domain at the sequence RPLPSPP in the region near its C terminus. Mutations within this sequence or at Tyr762 of p130Cas caused a significant reduction in the association of p130Cas with Src, and no association was detected when both of them were deleted. The kinase activity in v-Crk-transformed cells was also associated with p130Cas through this region. On the other hand, the deletion of the substrate domain abolished the binding with v-Crk. The association through the C-terminal region of p130Cas with Src kinase may facilitate effective hyper-phosphorylation of tyrosine residues in the substrate domain of p130Cas, resulting in the binding of SH2-containing molecules to p130Cas.

S src family cytoplasmic tyrosine kinases, including Src, Fyn, and Lyn, possess conserved, noncatalytic, and regulatory domains comprised of Src homology 2 (SH2) and Src homology 3 (SH3) domains. SH2 domains are composed of ~100 amino acids and specifically interact with sequences containing phosphotyrosine (1–3). SH3 domains are composed of ~50 amino acids and interact with proline-rich amino acid sequences (1–4). These SH2 and SH3 domains exert their functions, e.g. regulating the own kinase activity (5–7), connecting other signaling molecules to tyrosine kinases (8–10), and locating the proteins to the site of cytoskeleton (11), by inter- or intramolecular association with specific polypeptide sequences.

EXPERIMENTAL PROCEDURES

Cdk Lines and Antibodies—3Y1-Crk is an isolated clone of rat 3Y1 cells (30) transfected with v-crk cDNA (2) of an avian sarcoma virus.
CT10, inserted in an expression vector, pMV7-31, SR-3Y1 (32) is a 3Y1-derived cell line transformed by the v-Src allele of Rous sarcoma virus. Stable transformants of NIH 3T3 cells transfected with v-src (3T3-v-src) were established as described (18). Mouse monoclonal antibodies against phosphotyrosines (4G10) (33) were collected from culture supernatant of hybridoma cells. Anti-Cas2 is a polyclonal antibody against p130Cas (18) and an antibody against v-Crk protein and is described elsewhere (18). mAB 327 is a monoclonal antibody against Src (34) and is kindly provided by J. S. Brunger.

In Vitro Mutagenesis of p130Cas and Construction of Eukaryotic Expression Vectors—The short form of the cDNA of p130Cas (18) was cloned into M13mp19 vector. To make ΔSH3, ΔP1, and ΔSD constructs, several restriction sites, were introduced by site-directed mutagenesis method using an M13 in vitro mutagenesis kit (Bio-Rad). Deletion mutants were made by cutting out SalI fragments from mutated clones. The nucleotides corresponding to amino acid residues 640–642 were converted from PLL to LGS by site-directed mutagenesis to make the RPL* mutant. Tyr402, Tyr406, and both tyrosine residues are similarly mutated to phenylalanine to construct Y751F, Y762F, and Y751F/Y762F mutants, respectively. The ΔSB construct was made by cutting out a HindII fragment from p130Cas-cDNA. The wild-type and mutated p130Cas cDNAs were cloned into pS5Rα vector (35) to make eukaryotic expression vectors.

A eukaryotic GST (glutathione S-transferase) fusion expression vector, pEGB and pEGB-p230 (36) are generous gifts from B. J. Mayer. Mutations of the p130Cas moiety, described above, were also introduced into this vector.

Construction of Bacterial Expression Vectors for GST Fusion Proteins—GST fusion constructs of several subregions of p130Cas were constructed by subcloning fragments of p130Cas-cDNA into the Smal site of pGEX-3X vector. To make GST-SB and GST-RPL* constructs, PvuII fragments corresponding to residues 693–797 of wild-type p130Cas and 81–140 of RPL* were cloned into the SmaI site of pGEX-3X vector. To make GST-SrcSH2, the Xhol-MluI fragment of D mutant of chicken c-src (6) was blunt-ended with Klenow fragment of Escherichia coli and cloned into the Smal site of pGEX-1. For GST-CrkSH2 fusion protein, the SfiI-EcoBI fragment of v-crk was blunt-ended with the Klenow fragment of E. coli and ligated to the Smal site of pGEX-1. To generate GST-SrcSH3 protein and GST-CrkSH3 protein, oligonucleotides flanking SH3 domains of c-Src (residues 81–140) and v-Crk (residues 357–440) and introducing restriction sites were used for polymerase chain reaction, and the products were cloned into BamHI-EcoRI sites of pGEX-1 and pGEX-2T, respectively. GST-Grb2/AsHSH2 is a gift from Y. Takenawa.

In Vitro Binding Assays between GST Fusion Proteins—GST fusion proteins were expressed in E. coli and purified by affinity chromatography using immobilized glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.). One hundred twenty μg of the purified GST fusion proteins of domains of p130Cas were reacted with 20 μl of activated CNBr-Sepharose (Pharmacia), following the manufacturer’s instructions. Optical density of the supernatant of the reaction mixture at 260 nm was less than that of the C-form of p130Cas detected in 3Y1-Crk cells. An aliquot of 1% SDS. The eluate was mixed with 20 μl of 4× sample buffer (8% SDS, 0.4 μM Tris-HCl, pH 6.8, 10% glycerol, 0.04% bromphenol blue, and 0.4 μM dithiodithreitol) and analyzed on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining.

Transient Expression of Mutated p130Cas in COS-1 Cells or in 3T3 Cell Lines—COS-1 cells were transfected by the DEAE-dextran method. In brief, cells were cultured in a 15-cm dish to semiconfluent (approximately 2 × 106 cells/dish), and 50 μg of plasmid DNA was added with DEAE-dextran (4 mg/ml) and then cultured at 37 °C for 72 h. In the case of pS5Rα-S27F-c Src and pS5Rα-v-Crk, the added plasmid DNA was 5 and 25 μg, respectively. In the case of pS5Rα-S27F-c Src and pS5Rα-v-Crk, the added plasmid DNA was 5 and 25 μg, respectively. 3T3-v-Crk cells were transfected with 22.5 μg of plasmid DNA/15-cm dish using the CaPO4 method and cultured for 48 h.

Immunoprecipitation and Western Blotting—For protein analysis, cells were lysed in 1% Triton buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4). For immunoprecipitations, 250 μl of 3Y1 cell lysates or 100 μl of COS-1 cell lysates (approximately 4 μg/ml) were mixed with 5 μl of anti-Cas2 and incubated for 1 h at 4 °C. Samples were rotated with protein A-Sepharose (Sigma) for 1 h at 4 °C, and then the beads were washed four times with 1% Triton buffer and boiled in 1× sample buffer. Western blotting was performed as described (37) using anti-Cas2 (1:2500), 4G10 (5 μg IgG/ml) as first antibodies and detected by protein A-Western AP system (Pharmacia). In some cases, Western blotting was performed using anti-Cas2 (1:5000), 4G10 (0.5 μg/ml IgG), or mAB 327 (1:5000) as first antibodies and detected by ECL Western blotting analysis system (Amersham Corp.). Affinity Precipitation with GST Fusion Proteins—Bacterial lysates including approximately 200 μg of GST-SH2 or SH3 fusion proteins were reacted with glutathione-Sepharose 4B beads (Pharmacia), and the beads were incubated with cell lysates of transformed 3Y1 cells or transfected COS-1 cells. After 1 h of incubation at 4 °C, the beads were washed four times with 1% Triton buffer and boiled in 1× sample buffer. The proteins were separated on a 7.5% SDS-polyacrylamide gel. Western blotting was performed as described above.

In Vitro Kinase Reaction—3T3-v-Crk cells transfected with the mutants of p130Cas-cDNA were lysed in 1× Triton-X buffer and incubated with 20 μl of glutathione-Sepharose 4B beads for 1 h at 4 °C. The beads were washed four times with 1% Triton-X buffer and twice with kinase buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM MgCl2, 10 mM MnCl2) and resuspended in 30 μl of kinase buffer. Kinase reaction was performed with or without 20 μg of poly-Glu-Tyr (Sigma) at room temperature for 30 min. After adding 30 μl of 4× sample buffer, samples were separated by SDS-PAGE. Gels were fixed and incubated in 1 N KOH for 1 h at 55 °C to reduce backgrounds derived from phosphorylated serines and threonines, before autoradiography. Silver staining for checking the expression levels was performed as described (38).

RESULTS

In Vitro Association of SH2 and SH3 Domains of Src and Crk with p130Cas—GST fusion proteins containing the SH2 or SH3 domains of Src or Crk were constructed to determine which domains of Src and Crk are involved in the association with p130Cas. GST fusion proteins were immobilized on glutathione-Sepharose beads and incubated with lysates of 3Y1, 3Y1-Crk, and SR-3Y1. After washing the beads, affinity-purified proteins with GST-fusion proteins were subjected to SDS-PAGE and Western blotting using anti-Cas2. In normal 3Y1 cells, p130Cas can be detected as A and B forms (Fig. 1, lane 1), and in 3Y1-Crk cells, a broad band of the C form appears (Fig. 1, lane 3). The A and B forms are regarded as the tyrosine-unphosphorylated forms, and the C form that appears as a broad smear is regarded as the tyrosine-phosphorylated form, which includes various numbers of phosphorylated tyrosines (18, 19). Although marked tyrosine phosphorylation of p130Cas is detected in SR-3Y1 cells, the position of the phosphorylated band is lower than that of the C form of p130Cas detected in 3Y1-Crk cells (Fig. 1, lane 2) (18).

Both GST-SrcSH2 and GST-CrkSH2 bound to the C form of p130Cas but not to the A or B form (Fig. 1, lanes 4–6 and...
Binding Regions of p130Cas for Src and v-Crk

16–18). GST-SrcSH3 bound to the A, B, and C forms of p130Cas (Fig. 1, lanes 7–9). We could not detect the binding of GST-CrkSH3 to p130Cas (Fig. 1, lanes 19–21). Negative controls, GST and GST-Grb2AshSH2, did not bind p130Cas (Fig. 1, lanes 10–12 and 13–15). From these results, we concluded that the SH2 domains of Src and Crk are associated with p130Cas in a phosphorylation-dependent manner and that the Src SH3 domain can be associated with p130Cas in a phosphorylation-independent manner.

Association of the Src SH3 Domain with the C-terminal Proline-rich Region of p130Cas—The short form of p130Cas cDNA (18) was used for construction of a series of deletion mutants of p130Cas (Fig. 2A), since it could encode a 120-kDa gene product, which corresponds to the B form of p130Cas. These mutants were transiently expressed in COS-1 cells, and the cell lysates were reacted with immobilized GST-SrcSH3 fusion proteins to determine the binding site of the Src SH3 domain to p130Cas (Fig. 3A). GST-SrcSH3 failed to bind ∆SB mutant (Fig. 3A, lanes 11 and 12), although it bound the wild-type p130Cas (Fig. 3A, lanes 3 and 4) and the other deletion mutants including the ∆P1 mutant, which lacks a proline-rich region following the SH3 domain (Fig. 3A, lanes 5–10). The endogenous p130Cas was seen on longer exposure (data not shown).

The deleted region in ∆SB mutant contains the sequence RPLPSPPKF, corresponding to residues 733–741. This sequence is close to the class I consensus sequence, RXL PLLPRF (F represents a hydrophobic residue) for the Src SH3 domain (39, 40). A mutant RPLP*K (Fig. 2A), in which the RPLPSPPKF sequence was converted to RSLLGPPKF, was expressed in COS-1 cells. GST-SrcSH3 fusion protein failed to bind this mutant (Fig. 3B, lanes 19 and 20). Thus, we located the binding site of the Src SH3 domain to the RPLPSPPKF sequence of p130Cas. Furthermore, the W119A mutant of c-Src (6), which has an alanine residue instead of the tryptophan residue well conserved in various SH3 domains, failed to bind the wild-type p130Cas (Fig. 3B, lane 17), confirming that this tryptophan is essential to the binding of the Src SH3 domain with p130Cas.

In Vitro Binding of the Src SH3 Domain with GST-p130Cas

Fusion Proteins—To verify that the Src SH3 domain directly binds p130Cas, affinity between purified GST-SrcSH3 fusion protein and purified GST-fusion proteins containing various parts of p130Cas (Fig. 2B) was examined. GST-SB construct of p130Cas was shown to bind GST-SrcSH3 (Fig. 4, lane 3), indicating that the association between p130Cas and the Src SH3 domain is direct. RPLP* mutant did not bind GST-SrcSH3 (Fig. 4, lane 4), confirming that this site is involved in the binding.

Association between p130Cas and 527F-c-Src—To investigate the association of p130Cas with Src in vivo, the mutants of p130Cas and activated chicken c-Src (527F-c-Src) were co-expressed in COS-1 cells, and the lysates were immunoprecipitated with anti-Cas2 antibody. Each series of mutants, of which tyrosine residues were conserved to bind phosphorylated tyrosine residues, Tyr751 to Phe did not reduce the binding ability at all (Fig. 5A, lane 3–5, and 7–9). TY751F-c-Src was detected in the complex immunoprecipitated by 527F-c-Src, although the phosphorylation level of ∆SB mutant was hardly detectable (Fig. 5D, lane 2). The expression of 527F-c-Src was comparable among the samples (Fig. 5B). In the case of wild-type p130Cas, 527F-c-Src was detected in the complex immunoprecipitated with anti-Cas2 (Fig. 5A, lane 3); however, the ∆SB mutant and RPLP* mutant showed extremely reduced amounts of the associated Src in anti-Cas2 immunoprecipitants (Fig. 5A, lanes 2 and 4). These results suggest that the interaction through the Src SH3 domain should be critical for the association between both proteins in vivo.

To elucidate the interaction through the SH2 domain, another series of mutants, of which tyrosine residues were converted to phenylalanine residues, were constructed and co-expressed in COS-1 cells with 527F-c-Src. The mutation of Tyr762 of p130Cas to Phe reduced the binding ability to 527F-c-Src to less than one-third of that of the wild type (Fig. 5A, lane 6), but deletion of the substrate domain and the mutation of Tyr751 to Phe did not reduce the binding ability at all (Fig. 5A, lane 5). These results suggest that Tyr762 also contributes to the binding with 527F-c-Src. Since SH2 domains are considered to bind phosphorylated tyrosine residues, Tyr762 should be the binding site for the Src SH2 domain in vivo.
Binding Regions of p130Cas for Src and v-Crk

Association between p130Cas and v-Crk Expressed in COS-1 Cells—The substrate domain of p130Cas has repetitive YXXP motifs that are estimated to be the binding sites of v-Crk. We co-expressed v-Crk and mutants of p130Cas in COS-1 cells to determine the binding sites of v-Crk. We used the eukaryotic GST expression system to generate mutants of p130Cas fused to GST (36) and precipitated the mutants with glutathione-Sepharose beads, since immunoprecipitation can mask the band corresponding to v-Crk by heavy chains of immunoglobulins on Western blots. GST-fusion proteins of wild-type p130Cas and ΔSD mutant were expressed at similar protein levels (Fig. 6C), and the expression levels of v-Crk were comparable (Fig. 6B). Although the wild-type p130Cas bound v-Crk (Fig. 6A, lane 2), no association of v-Crk was detected with ΔSD mutant (Fig. 6A, lane 3), suggesting that v-Crk binds to the substrate domain of p130Cas.

The Domain of p130Cas with the Kinase Activity in v-Crk-Transformed Cells—To determine whether the C-terminal Src binding domain also plays a role in phosphorylating p130Cas in the process of the transformation by v-Crk, and to get insight into the kinase responsible for the phosphorylation of p130Cas in v-Crk-transformed cells, we investigated the in vitro kinase activity associated with the mutants of p130Cas expressed in 3T3-v-Crk cells (18). To discriminate expressed mutants from endogenous p130Cas, GST-fusion proteins of the mutants of p130Cas were expressed in 3T3-v-Crk cells. Lysates of cells transfected with the plasmids encoding the wild-type p130Cas and the mutants were precipitated with glutathione-Sepharose beads and subjected to the kinase reaction with or without poly-Glu-Tyr as substrates (Fig. 7A). The wild-type p130Cas showed phosphorylation of itself and of poly-Glu-Tyr (Fig. 7A, lanes 2 and 7), suggesting that the kinase activity is associated with p130Cas in these cells. RPLP* mutant, in which the SH3 binding site is destroyed, was associated with markedly reduced kinase activity (Fig. 7A, lanes 3 and 8), while in...
Y762F mutant, associated kinase activity was comparable with that in the cells expressing wild-type p130Cas (lanes 2 and 4). 

D SB mutant, which lacks a region containing both SH2 and SH3 binding sites for activated c-Src, was associated with completely reduced kinase activity (Fig. 7A, lanes 5 and 6). Although the kinase(s) that phosphorylates p130Cas in v-Crk-transformed cells is not known, this result shows that the SH3 binding site of p130Cas is associated with the kinase activity, which can phosphorylate p130Cas at least in vitro, in the 3T3-v-Crk cells.

**DISCUSSION**

p130Cas is a phosphoprotein that has characteristic, clustered, and repeated (I/V/L)YXXP motifs (Table I) in its "substrate domain" (18) and is supposed to be an ideal substrate for tyrosine kinases including Src family kinases and Abl (25). Furthermore, these motifs conform very well to the consensus binding sequence for the Src SH2 domain. p130Cas has also a proline-rich region and several tyrosine residues near its C terminus, suggesting that this region could provide the binding sites for the SH2 and SH3 domains. Here we report that both SH2 and SH3 domains of Src tyrosine kinase bind to the C-terminal region of p130Cas, whereas the v-Crk binds to the substrate domain through the SH2 domain.

We revealed that Tyr762 of p130Cas is one of the binding sites for Src. As SH2 domains are thought to interact with phosphorylated tyrosine, Tyr762 is estimated to be the binding site for the Src SH2 domain. The sequence around Tyr762 (Table I) is similar to the consensus sequence for the Src SH2 domain determined by the phosphopeptide library (26) and has a hydrophobic amino acid, valine, at the +3 position. A phosphorylated tyrosine and a hydrophobic amino acid residue at the +3 position are considered to be important for the binding between SH2 domains and tyrosine-containing peptides (41).

The binding sequence for the Src SH3 domain is a RPLPSPKFK sequence corresponding with amino acid residues 733–741 of p130Cas. The association between purified GST-fusion proteins suggests that the interaction between the Src SH3 domain and p130Cas is direct and does not require any intermediate proteins. The RPLPSPP sequence of p130Cas matches the Class I consensus sequence for the Src SH3 domain (Table II) determined by a biased random peptide library (39, 40). The known ligands for the Src SH3 domain are shown in Table II (8, 42–44), including the RPLPSPKFK sequence of p130Cas.

So far, three substrates for Src family kinase, AFAP-110 (11, 24), Sam68 (9, 10), and GAP-associated p62 (29, 45) were reported to interact with Src family kinase through both SH2 and SH3 domains. In these cases, mutants of Src family kinases in the SH3 domains could not tyrosine-phosphorylate these substrates (9, 24, 45). Therefore, the interaction through SH3 domains is assumed to be involved in the substrate recognition by these kinases. In the case of p130Cas, although a mutant in the SH3 domain of Src, which had an impaired SH3 binding ability, could tyrosine-phosphorylate p130, the phosphorylation level was reported to be low (24). This fact suggests that the Src SH3 domain plays a role in phosphorylating p130Cas. In this report, we show that the Src SH3 domain binds p130Cas. Furthermore, in the co-expression system of COS-1...
cells, the mutant that destroyed only the RPLPSPP sequence showed a certain level of phosphorylation; however, the mobility shift may be the result of a low level of phosphorylation. We also revealed that the tyrosine kinase activity for poly-Glu-Tyr and p130Cas itself was associated with the RPLPSPPKF sequence of p130Cas in 3T3-v-Crk cells. These results suggest that the binding through the SH3 domain would be important in tyrosine-phosphorylating p130Cas.

In our model, the binding through the Src SH3 domain is thought to have a role in substrate recognition before tyrosine phosphorylation, and the Src SH2 domain reinforces the binding after tyrosine phosphorylation. As a result, the two-site binding interaction creates a strong association between Src and p130Cas. This tight association may cause the effective hyperphosphorylation of p130Cas by Src tyrosine kinase. In the association between c-Src and p130Cas, p130Cas might open the "closed form" of c-Src by binding to the regulatory domain and up-regulate the kinase activity of c-Src. This possibility still remains under investigation now. Tyrosine phosphorylation of p130Cas should allow the recruitment of SH2-domain-containing signaling molecules such as c-Crk (46) or Nck (47) and their associated proteins. This might enable these proteins to be tyrosine-phosphorylated by Src or to interact with the molecules associated with the SH3 domain of p130Cas. Thus, p130Cas may serve as a "docking protein" linking Src to downstream signaling molecules. To clarify the role of p130Cas in transformation, we are now searching the molecules that comprise the complex with p130Cas.

Although we revealed that the W119A mutant of the Src SH3 domain fails to bind p130Cas, the W119A mutant of p27F-c-Src had a full transforming activity compared with parental 527F-c-Src (6, 7). At present, we have no evidence to tell whether the binding of p130Cas to the Src SH3 domain controls the kinase activity of Src positively or negatively. There are several proteins that bind the Src SH3 domains other than p130Cas. The cooperative roles of these molecules on the regulation of the transforming activity of Src oncprotein should be elucidated.

In the transformation by v-Crk, the tyrosine kinase that phosphorylates p130Cas is not known. In this report, we reveal that most of the tyrosine kinase activity in v-Crk-transformed cells is also associated with the Src-binding C-terminal region of p130Cas, especially the RPLPSPP sequence. This suggests that the binding of some kinases(s) to this sequence through the SH3 domain like Src kinase plays a role in phosphorylating p130Cas. Formerly, it was reported that v-Crk caused the elevation of the co-expressed c-Src kinase activity in 3Y1 cells (48). In our previous reports, co-overexpression of c-Src in v-Crk-transformed NIH 3T3 cells raised the tyrosine phosphorylation of p130Cas (18), and p130Cas could be a substrate for c-Src in vitro (19), suggesting that c-Src might be the kinase to

phosphorylate p130Cas. We showed here that the p130Cas-associated tyrosine kinase activity in v-Crk-transformed cells is mostly associated with the Src-binding region of p130Cas whereas v-Crk binds the substrate domain of p130Cas. Therefore, the kinase activity that we see here is more likely to be caused by some other kinases that bind the Src-binding region of p130Cas rather than by v-Crk-associated kinases such as Abl (36, 49, 50). However, our data cannot exclude the possibility that some tyrosine kinase that does not form a stable complex with p130Cas phosphorylates p130Cas in 3T3-v-Crk cells. We are now searching for the kinase that tyrosine-phosphorylates p130Cas in v-Crk-transformed cells. Furthermore, the mechanism by which v-Crk activates the tyrosine kinase and the pathway through which the phosphorylation of p130Cas is involved in the cell transformation should be elucidated.

Our results give an insight into the mechanism of signal transduction in v-Src- and v-Crk-transformed cells and also provide information on the physiological role of unphosphorylated p130Cas as a partner of tyrosine kinase.

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Table II: Sequence alignment of the Src SH3 binding motifs

| Class 1 consensus | Src library consensus | p130Cas SH3 motif |
|-------------------|----------------------|------------------|
| X                  | P                    | P                |
| S                  | R                    | L                |
| L                  | P                    | P                |
| I                  | F                    | P                |
| E                  | R                    | F                |
| S                  | P                    | X                |
| P                  | S                    | F                |

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| I                  | F                    | P                |
| E                  | R                    | F                |
| S                  | P                    | X                |
| P                  | S                    | F                |
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