Characterization and Binding Specificity of the Monomeric STAT3-SH2 Domain

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Signal transducers and activators of transcription (STATs) are important mediators of cytokine signal transduction. STAT factors are recruited to phosphotyrosine-containing motifs of activated receptor chains via their SH2 domains. The subsequent tyrosine phosphorylation of the STATs leads to their dissociation from the receptor, dimerization, and translocation to the nucleus. Here we describe the expression, purification, and refolding of the STAT3-SH2 domain. Proper folding of the isolated protein was proven by circular dichroism and fluorescence spectroscopy. The STAT3-SH2 domain undergoes a conformational change upon dimerization. Using an enzyme-linked immunosorbent assay we demonstrate that the monomeric domain binds specific phosphotyrosine peptides. Furthermore, we get the proteins to specific phosphotyrosyl peptide sequences within their binding partners, thereby regulating a wide range of transcription factors (1, 2). These noncatalytic domains target the phosphotyrosyl subunit of phosphatidylinositol 3-kinase, and the STAT family for the amino-terminally His-tagged STAT3-SH2 domain was verified for the binding of STAT monomers to the phosphotyrosine-containing tail segments.

Previous experiments have shown that the SH2 domain is also the sole determinant of specific STAT factor activation via gp130 and the interferon-γ receptor (16, 17). The mechanism for the binding of STAT monomers to the phosphotyrosine-containing recruitment sites of the cytoplasmic region of signal-transducing receptor subunits still needs to be elucidated.

Here we describe the expression, refolding, and structural characterization of the STAT3-SH2 domain as well as its specific binding to phosphotyrosine peptides. Furthermore, we demonstrate that this interaction requires a monomeric domain.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Biotinylated peptides were synthesized as described earlier (18).

Plasmid Construction—Constructions were carried out using standard procedures (19). For construction of p8-STAT3-BS, the cDNA encoding murine STAT3 (kindly supplied by J. Darnell, Jr., Rockefeller University, New York) was provided with BglII and SalI restriction sites at the 5’- and 3’-ends, respectively, and cloned into a pBluescript vector (Stratagene, Heidelberg, Germany). The sequence encoding the STAT3-SH2 domain (amino acid residues 582–702) was amplified by polymerase chain reaction, and BamHI and AvaII restriction sites were introduced by the 5’- and 3’-primers, respectively. The BamHI/AvaII DNA fragment was ligated with a modified pRSetEc vector carrying an adaptor consisting of an amino-terminal MRGS(H)₆-tag and a BamHI and an AvaII restriction site. The resulting vector pRSetS3SH2 coding for the amino-terminally His-tagged STAT3-SH2 domain was verified by DNA sequence analysis.

Expression, Purification, and Refolding of the Recombinant MRGS(H)₆-tagged STAT3-SH2 Domain—Escherichia coli strain BL21(DE3)pLysS transformed with the pSetS3SH2 plasmid was grown at 37 °C in LB medium containing chloramphenicol (50 μg/ml) and ampicillin (100 μg/ml) to an A₅₉₅ of 0.6–0.7. Cells were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C and subsequently harvested by centrifugation. The bacterial pellet was suspended in lysis buffer (26 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM tyrosine motifs within the interleukin-6 signal-transducing receptor subunit gp130 have been identified (6, 7). Two of these motifs (Y767RHQ and Y814FKQ) give rise to specific STAT3 activation, whereas two others (Y905LPQ and Y915MPQ) are able to recruit both STAT1 and STAT3 (6). Subsequent to receptor binding, the STAT factors are phosphorylated on a single tyrosine residue by receptor-associated tyrosine kinases of the Janus kinase family (8–10). This activation of the STAT factors leads to homo- or heterodimerization and translocation to the nucleus, where they bind to enhancers of interleukin-6-inducible genes resulting in the activation of transcription of, e.g. acute phase protein genes (11–13). The dimerization of STAT factors has also been shown to be mediated by the SH2 domains (9). This has been confirmed recently by x-ray structures of the STAT1 and STAT3 dimers bound to DNA (14, 15). In this complex the two SH2 domains form a tunnel that is passed by the two phosphotyrosine-containing tail segments.

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phenethylsulfonyl fluoride, 1 mM dithiothreitol), cells were lysed by repeated freezing and thawing, and the inclusion bodies were harvested by centrifugation and purified by five cycles of sonication (constant pulse for 2 min at 0 °C), each followed by centrifugation at 3,700 rpm for 30 min. The inclusion bodies were solubilized in buffer S (50 mM Tris-HCl, pH 8.0, 6 M guanidine HCl, 1 mM EDTA, 100 mM dithiothreitol). After incubation at 42 °C for 30 min, insoluble particles were removed by filtration through a 0.45-μm sterile filter, and the buffer pH was adjusted to 2. The denatured STAT3-SH2 domain was purified by reverse phase HPLC using a Polyosil 60-5 C18 column (CS-Chromatographic Service GmbH, Langerwehe, Germany) equilibrated in buffer A (0.1% trifluoroacetic acid). Elution of the STAT3-SH2 domain occurred at 50.3% buffer B (80% acetonitrile, 0.1% trifluoroacetic acid). The purified protein was isolated by lyophilization and solubilized in buffer S. Refolding of the purified SH2 domain was achieved by dialysis at 4 °C against different buffers (buffer C: 20 mM NaHPO4/KH2PO4, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol; buffer D: 20 mM NaHPO4/KH2PO4, pH 5.5, 150 mM NaCl, 0.5 mM EDTA; buffer E: 20 mM CH3COONa/CH3COOH, pH 4.5, 150 mM NaCl, 0.5 mM EDTA). For purity determination, the proteins were resolved on a 15% polyacrylamide gel by SDS-PAGE and visualized using SyproTm Orange protein stain reagent (Molecular Probes, Eugene, OR). The quantitative analysis was performed on a Storm 840 fluorescence scanner (Molecular Dynamics, Krefeld, Germany) using Image QuantTm software.

Immunoblot Analysis—The harvested cell fragments containing the inclusion bodies were resolved by SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) using a semidry electroblotting apparatus. STAT3-SH2 detection was performed using a monoclonal MBiHis antibody (QIAGEN, Hilden, Germany). A polyclonal goat anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, Hamburg, Germany) was used to visualize the immunoreactive bands by Western blot techniques.

Peptide Binding Assay—Peptide binding of the isolated pure STAT3-SH2 domain was performed by the means of an ELISA. A 96-well ELISA Maxisorb plate (NUNC, Roskilde, Denmark) was coated with 2.5 μg/ml streptavidin (biotinylated peptides were immobilized by incubating the streptavidin surface with 100 μg/ml streptavidin). After incubation of the surface with 100 μg/ml/well phosphate-buffered saline and 0.02% Tween, and unbound protein was removed by washing four times with the appropriate phosphate buffer. The time constant ranged between 1 and 4 s and the cell path length was 15 mm. The spectral band width was 1.5 nm. The excitation wavelength was 295 nm. The spectra are corrected for changes in lamp intensity and for spectral sensitivity of the emission-monochromator/photonmultiplier system. All fluorescence measurements were carried out at 20 °C.

Fluorescence lifetimes and anisotropy decay were measured in the single photon-counting mode with an Edinburgh Instruments Ltd. (U. K.) spectrometer, model 199. The full width at half maximum of the lamp pulse from the hydrogen flashlamp was 1.4 ns. The excitation wavelength was 295 nm and the band width 8 nm. The emitted light was passed through a combination of a UV-transmitting black glass and a cutoff glass filter to create a band pass (WG320, DUG11, Schott, Mainz, Germany). At least 80,000 counts were accumulated in the peak channel of the total fluorescence intensity, I(t).

Circular Dichroism Spectroscopy—CD measurements were carried out on an AVIV (Lakewood, NJ) 62DS CD spectrometer, equipped with a temperature control unit, and a Jasco J-600 spectropolarimeter, both calibrated with a 0.1% aqueous solution of p-toluenesulfonic acid according to Chen and Yang (20). The spectral band width was 1.5 nm. The time constant ranged between 1 and 4 s and the cell path length was between 0.1 and 10 mm.

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded on a Spex Fluorolog 211 photon-counting spectrofluorometer (Spex Industries, NY) with a band width of 2.7 nm (excitation monochromator) and 2.2 nm (emission monochromator). The excitation wavelength was 295 nm. The spectra are corrected for changes in lamp intensity and for spectral sensitivity of the emission-monochromator/photonmultiplier system. All fluorescence measurements were carried out at 20 °C.

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Fluorescence anisotropy decays were analyzed by an exponential fit.

\[ r(t) = \exp(-\phi \cdot t) + r_r \]

where \( r = r_0 + r_r \). The parameters of r(t) are as follows. r is the anisotropy, \( \phi \), the rotational correlation time; \( r_0 \) and \( r_r \) are the limiting anisotropies, \( r(t \to 0) = r_0 \) and \( r(t \to \infty) = r_r \). The quality of the fits was gathered from plots of weighted residuals and from the statistical parameter \( x^2 \).
from absorption spectra in the range of 240–320 nm using the method of Waxman et al. (22).

RESULTS
Expression and Purification of Recombinant STAT3-SH2—To obtain sufficient amounts of protein, the amino-terminally His-tagged STAT3-SH2 domain was expressed in *E. coli*. The recombinant protein was found entirely in inclusion bodies (Fig. 1A). Repeated sonication and centrifugation yielded inclusion bodies containing about 90% STAT3-SH2 protein. 1 liter of medium contained 40–50 mg of inclusion body proteins. After solubilization of the inclusion bodies in GdnHCl the proteins were separated on a reverse phase HPLC column, and STAT3-SH2-containing peak fractions were lyophilized. The STAT3-SH2 protein proved to be at least 99% pure (Fig. 1B). This procedure yielded 10–15 mg of pure STAT3-SH2/liter of culture.

Refolding and CD Spectroscopy—The purified protein was dissolved in 6 M GdnHCl, 1 mM EDTA, and 100 mM dithiothreitol and dialyzed for refolding against buffers of pH 7.5, 5.5, and 4.5, respectively. Subsequently the protein samples were characterized by CD spectroscopy. Fig. 2 shows the far UV and near UV CD spectra of the STAT3-SH2 domain at pH 7.5 (solid line) and pH 4.5 (dashed line). Although the far UV CD spectra are remarkably different at the two pH values, they look similar to spectra of other SH2 domains (23, 24). Even more pronounced differences were detected between the near UV CD spectra at the two different pH values (Fig. 2B). For instance, at pH 4.5 a distinct band appeared at 292 nm which can be assigned to a tryptophan residue. This effect can be attributed to a local change rather than to a change of the overall fold of the protein. The reversibility of this structural change was determined by changing the pH of the solution from pH 4.5 to 7.5 and vice versa (data not shown).

To determine the thermal stability of the folded proteins at the different pH values we recorded a series of CD spectra with increasing temperature. At pH 7.5 a melting curve with a midpoint around 43 °C was obtained (Fig. 3). At pH 4.5, however, the protein precipitated with increasing temperature (data not shown).

Unfolding by GdnHCl—Because the thermal stability of the protein could only be estimated at pH 7.5, we monitored the GdnHCl-induced unfolding of the protein by fluorescence spec-
Fluorescence Spectroscopy—Steady-state fluorescence, fluorescence lifetime, and anisotropy decay measurements were performed at pH 7.5, 5.5, and 4.5. The steady-state fluorescence data of the SH2 domain upon excitation at 295 nm are compiled in Table I. With a pH decrease from 7.5 to 4.5, the maximum of the emission band shifted from 336 to 330 nm. This shift was accompanied by a decrease of the full width at half maximum from 56 to 51 nm. The shift of the emission maximum and the decrease of the full width at half maximum are indicative of an increasingly hydrophobic environment of the sole tryptophan present in the SH2 domain.

The results of the fluorescence lifetime and anisotropy decay measurements are compiled in Tables II and III. The decays of the tryptophan fluorescence can be fitted by a sum of three exponentials with fractional intensities $B_i$ and corresponding lifetimes $\tau_i$ and lead to calculated mean lifetimes ($<\tau>$) of 4.7, 4.1, and 3.7 ns for the pH values of 7.5, 5.5, and 4.5, respectively (Table II). Such a behavior is in good agreement with the blue shift of the emission maximum in Table I (25).

The fluorescence anisotropy decays of the SH2 domain at

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**FIG. 3.** Thermal stability of the refolded STAT3-SH2 domain at pH 7.5. Thermal stability was determined by CD spectroscopy. The graph shows the ellipticity $\theta_{\text{MRW}}$ (MRW, mean residue weight) at 215 nm as a function of temperature.

**FIG. 4.** Unfolding of the recombinant STAT3-SH2 domain. The figure shows unfolding of the STAT3-SH2 domain by increasing concentrations of GdnHCl at pH 4.5 ($\cdot$, dashed line), pH 5.5 ($\circ$, dotted line) and pH 7.5 ($\triangle$, solid line). The wavelength shift of maximum tryptophan emission $\lambda_{\text{max}}$ was monitored as a function of GdnHCl concentration. Because $\lambda_{\text{max}}$ also changes with pH, the data were normalized. The $\lambda_{\text{max}}$ values observed at GdnHCl concentrations of 0 and 4 M were taken as representative of the 100% native and 0% native, i.e. fully denatured state, respectively.

**TABLE I**

| pH  | $\lambda_{\text{max}}$ | FWHM |
|-----|------------------------|------|
| 7.5 | 336                    | 56   |
| 5.5 | 334                    | 53   |
| 4.5 | 330                    | 51   |

$\lambda_{\text{max}}$ is the wavelength of the maximum tryptophan fluorescence. FWHM is the full width at half maximum.

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different pH values were fitted with one exponential and led to rotational correlation times of $\Phi = 12.4, 6.4,$ and $6.1$ ns at pH values of $7.5, 5.5,$ and $4.5$, respectively (Table III). Rotational correlation times $\Phi$ can be used to calculate the molecular mass from the equation $M_r = f \times \Phi (f = 2.6 \text{ kDa/ns})$ for spherical particles on the basis of the Stokes-Einstein relationship (26). The expected rotational correlation time $\Phi$ for a monomeric SH2 domain is therefore about $5.7$ ns assuming a spherical shape. The measured rotational correlation times at acidic pH values are in good agreement with the overall tumbling rate expected for a monomer. The twice as high value found at neutral pH indicates the existence of a dimeric SH2 domain. The reversibility of the monomer/dimer transition was determined by changing the pH from 4.5 to 7.5 and vice versa by dialysis (data not shown).

**Size Exclusion Chromatography—**Additional evidence for the dimerization was provided by size exclusion chromatography experiments (Fig. 5) using a calibrated column. Fig. 5A shows the elution profile of the SH2 domain (monomer, dotted line; dimer, solid line). Both peaks contain STAT3-SH2 as shown by SDS-PAGE (see inset in Fig. 5A). The apparent molecular masses of both the monomer (13 kDa) and the dimer (32 kDa) are in good agreement with the expected values (Fig. 5B) of 15 and 30 kDa, respectively.

**Specific Interaction of the Recombinant STAT3-SH2 Domain with Phosphopeptides—**To determine the functionality of the recombinant STAT3-SH2 domain, we worked out an ELISA using biotinylated phosphopeptides as bait. Based on previous investigations, we chose all of the phosphopeptide motifs of the signal transducer gp130, a mutant of the pY767 motif containing the amino acids of the gp130 motif pYQ770E (gp130-Y767) as well as the phosphotyrosine motif encompassing pY705 of STAT3 or a peptide containing the amino acids of the gp130 motif pYQ770E in random order (pYX) (Table IV). As the STAT3-SH2 domain turned out to undergo a pH-dependent dimerization, we investigated the specificity of the interaction with the various phosphopeptide motifs under neutral (dimer) and acidic (monomer) conditions. For the interaction of the peptides with the monomeric SH2 domain, we performed the assay at pH 5.5 to maintain the stability of streptavidin.

Fig. 6 shows a schematic representation of the ELISA used. After incubation of the biotinylated phosphopeptides with the streptavidin-coated surface, the immobilized phosphotyrosine residues were detected with the phosphotyrosine antibody PY20 (Fig. 6A). Incubation with increasing amounts of STAT3-SH2 led to a decrease in absorbance because PY20 was unable to recognize the phosphopeptides bound to the SH2 domain (Fig. 6B). The relative decrease in absorbance with increasing

| Peptide Origin and Y location | Sequence |
|-------------------------------|----------|
| pY<sup>963</sup> | gp130-Y<sup>963</sup> |
| pY<sup>759</sup> | gp130-Y<sup>759</sup> |
| pY<sup>787</sup> | gp130-Y<sup>787</sup> |
| pY<sup>214</sup> | gp130-Y<sup>214</sup> |
| pY<sup>1205</sup> | gp130-Y<sup>1205</sup> |
| pY<sup>415</sup> | gp130-Y<sup>415</sup> |
| pY<sup>Q770E</sup> | (gp130-Y<sup>787</sup>) |
| pY<sup>705</sup> | STAT3-Y<sup>705</sup> |
| pY<sup>8</sup> | |

For the interaction of the peptides with the STAT3-SH2 domain under monomeric (pH 5.5, dotted line) and dimeric (pH 7.5, solid line) conditions. The elution profile of the marker proteins (a, bovine thyroglobulin, 670 kDa; b, bovine γ-globulin, 158 kDa; c, chicken ovalbumin, 44 kDa; d, horse myoglobin, 17 kDa; e, vitamin B<sub>12</sub>, 1.35 kDa) is drawn as a dashed line. A and 0 denote the STAT-SH2 domain eluted at pH 7.5 and 5.5, respectively. The inset displays a SDS-PAGE analysis of fractions 3–13 showing STAT3-SH2. Protein bands were visualized by silver staining. The marker proteins b, c, and d were used for linear regression. B, plot of the log($M_r$) as a function of the retention times of the marker proteins (a–d). Elution times of the STAT3-SH2 domain at pH 7.5 (A) and 5.5 (C) correspond to molecular masses of 32 and 13 kDa, respectively.

**TABLE I**

F[ig. 5. **Size exclusion chromatography.** A, elution profile of the STAT3-SH2 domain at neutral pH (random order) and 5.5 (dotted line) and dimeric (pH 7.5, solid line) conditions. The elution profile of the marker proteins (a, bovine thyroglobulin, 670 kDa; b, bovine γ-globulin, 158 kDa; c, chicken ovalbumin, 44 kDa; d, horse myoglobin, 17 kDa; e, vitamin B<sub>12</sub>, 1.35 kDa) is drawn as a dashed line. A and 0 denote the STAT-SH2 domain eluted at pH 7.5 and 5.5, respectively. The inset displays a SDS-PAGE analysis of fractions 3–13 showing STAT3-SH2. Protein bands were visualized by silver staining. The marker proteins b, c, and d were used for linear regression. B, plot of the log($M_r$) as a function of the retention times of the marker proteins (a–d). Elution times of the STAT3-SH2 domain at pH 7.5 (A) and 5.5 (C) correspond to molecular masses of 32 and 13 kDa, respectively.

**TABLE II**

Fluorescence intensity decay data of the STAT3-SH2 domain at different pH values

| pH  | $B_1$ | $B_2$ | $B_3$ | $r_1$ | $r_2$ | $r_3$ | $t_1$ | $t_2$ | $t_3$ | $\chi^2$ | $\tau$ |
|-----|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|------|
| 7.5 | 0.19  | 0.64  | 0.17  | 1.34  | 4.54  | 9.25  | 1.41  | 4.7   |
| 5.5 | 0.18  | 0.59  | 0.23  | 1.05  | 3.55  | 7.61  | 1.10  | 4.1   |
| 4.5 | 0.17  | 0.62  | 0.21  | 0.94  | 3.31  | 7.15  | 1.23  | 3.7   |

**TABLE III**

Fluorescence anisotropy decay data of the STAT3-SH2 domain at different pH values

| pH  | $r_0$ | $r_1$ | $r_2$ | $\Phi$ | $\chi^2$ |
|-----|-------|-------|-------|-------|---------|
| 7.5 | 0.155 | 0.088 | 0.067 | 12.4  | 1.05    |
| 5.5 | 0.156 | 0.071 | 0.085 | 6.4   | 1.31    |
| 4.5 | 0.150 | 0.065 | 0.086 | 6.1   | 0.99    |

**TABLE IV**

Biotinylated peptides used in the ELISA
amounts of STAT3-SH2 compared with an SH2-free sample was used to determine the inhibition of the PY20/phosphopeptide interaction by STAT3-SH2 which indicates SH2/peptide binding.

Fig. 7A shows the inhibition of the PY20/phosphopeptide interaction at pH 5.5. The STAT3-SH2 domain interacts specifically with the four-membrane distal phosphotyrosine motifs of gp130 (pY767, pY814, pY905, and pY915) whereas the peptides pY683 and pYX as well as the pY759 motif, known to bind to the SH2 domain of SHP-2, show only weak binding. In addition, the isolated STAT3-SH2 domain binds to the motif pY705 of STAT3. Interestingly, the binding of the SH2 domain to pY767 is significantly impaired by a Q/E exchange at the pY position (pYQ770E). This emphasizes the importance of the pY+3 position for specific recognition of receptor motifs by STAT3-SH2.

Fig. 7B shows the results of the same experiment under neutral buffer conditions (pH 7.5) where the SH2 domain exists as a dimer. Whereas the monomeric SH2 domain is able to distinguish between the different phosphopeptides, the dimeric molecule is not. The phosphopeptides show only low affinity to the dimer.

DISCUSSION

The SH2 domains of the STAT factors are involved in receptor recognition as well as in their dimerization. Dimerization is induced by tyrosine phosphorylation and is a prerequisite for DNA binding. To investigate the interaction of the SH2 domains with the respective phosphotyrosine motifs on a molecular level we expressed the SH2 domain of STAT3 in E. coli. After purification the protein was refolded. CD spectra of the refolded protein correspond to those observed with other SH2 domains (23, 24). Spectral differences were observed at neutral and acidic pH. The changes seen in the far UV are a reflection of limited rearrangements of the overall structure (Fig. 2). The near UV spectrum, for instance, shows a distinct band at 292 nm at pH 4.5 which is not detectable at pH 7.5, indicating a loss in conformational mobility for the side chain of the sole tryptophan. This result correlates well with the corresponding fluorescence spectra where a blue shift of the emission maximum and a decrease of the full width at half maximum are observed with decreasing pH, reflecting a transition of the sole tryptophan from a hydrophilic to a more hydrophobic environment.

The fluorescence anisotropy decay measurements enabled us to assign these spectral differences to a pH-dependent dimerization of the recombinant SH2 domain which exists as a monomer under acidic conditions and as a dimer at neutral pH. The less exposed tryptophan in the monomeric state correlates with a higher stability of the monomer compared with the dimer as revealed by GdnHCl-induced denaturation. Thus, the small structural changes induced by dimerization are accompanied by a destabilization of the molecule.

Taken together, the fluorescence and CD measurements show that dimerization leads to a conformational change in the SH2 domain involving tryptophan 623. In the x-ray structure of the (STAT3)-2-DNA complex the corresponding tryptophan is located at the surface of the molecule and is accessible to water. The higher B factors of this amino acid residue in the crystal structure are a further indication of its enhanced flexibility in the dimer. For other SH2 domains such as those of Src or Lck kinase it has been shown that the BG loop is attached to the body of the molecule (27, 28), whereas it is completely detached in the (STAT3)-DNA and (STAT1)-DNA complexes (14, 15). Because the BG loop is also involved in the dimer interface we raise the idea that in the monomeric state this loop resembles the situation seen in other SH2 domains. This would bury the tryptophan (Trp623) within the structure, a fact that we indeed observe for the monomeric SH2 domain. The two conformational states might reflect different modes of SH2/phosphotyrosine peptide interactions in STAT receptor binding and STAT dimer formation.

Recently, heterodimeric complexes of STAT1 with STAT2 or STAT3 prior to cytokine stimulation have been described (29). The ability of the STAT3-SH2 domain to form dimers may reflect such an interaction between unphosphorylated STAT molecules. On the other hand, it cannot be ruled out that the observed dimerization is a property of the isolated domain and that its formation is prevented within the entire
protein.

To study the interaction of the STAT3-SH2 domain with different phosphotyrosine peptides we established an ELISA based on the competition of a phosphotyrosine monoclonal antibody with the recombinant protein in binding to phosphotyrosine peptide motifs. Whereas the monomeric STAT3-SH2 domain was able to bind to specific phosphotyrosine peptides no such interaction could be observed with the dimeric protein.

We detected a specific interaction between the monomeric STAT3-SH2 domain and the four distal phosphotyrosine motifs present in the cytoplasmic part of the signal transducer gp130. In contrast, the phosphotyrosine residues corresponding to the two membrane-proximal tyrosine residues did not show a specific interaction (Fig. 7). These results are in good agreement with the previous observation that in transiently transfected COS cells STAT3 is activated only through the four distal tyrosine motifs of gp130 (6, 7). Interestingly, we found a Q770E substitution in the Y767 motif of gp130 to lead to a loss in STAT3 activation (Fig. 7). Thus, our STAT3-SH2/phosphopeptide interaction studies fully confirmed the results obtained with native STAT3 in COS cells. Furthermore, the phosphotyrosine peptide of STAT3 itself (pY705) showed specific binding to the recombinant STAT3-SH2 domain. A comparison of the affinities would require a common binding mechanism. As deduced from the x-ray structure of the (STAT1)_2-DNA and (STAT3)_2-DNA complexes the mode of SH2/p peptide binding therein shows fundamental differences to SH2/peptide interactions known so far (2). An expected higher affinity to the phosphotyrosine peptide of STAT3 itself (pY705) compared with receptor motifs was not observed. This is presumably because the complex interaction seen in the x-ray structures cannot be reconstructed in the ELISA.

Thus far, structure/function studies of recombinant STAT3-SH2 domains were hampered by the fact that they did not show specific binding to phosphotyrosine peptides. We found that at physiological pH the recombinant STAT3-SH2 domain is forming dimers that do not bind to phosphopeptides. The observation that under acidic conditions, the STAT3-SH2 domain exists as a monomer that specifically binds to phosphotyrosine peptide motifs will enable us to elucidate how STAT factors interact with their receptors.

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