An Efficient Screening Assay for the Rapid and Precise Determination of Affinities between Leucine Zipper Domains†

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ABSTRACT: The protein products of the jun and fos oncogenes require a functional protein–protein interaction domain, called the "leucine zipper domain", to exert their transcriptional regulatory activity. A scintillation proximity assay was developed in which the biotinylated leucine zipper domain of the Jun protein (275–315) was immobilized on streptavidin-coated microfluorospheres and in which the leucine zipper domain of the Fos protein (160–200) was used as free, labeled ligand. The Fos leucine zipper peptide specifically bound to the Jun leucine zipper peptide, and for the first time, a dissociation constant (K_d = 110 ± 12 nM in PBS/0.1% Tween) could be determined. Optimal heterodimer formation was reached at neutral pH. Both acidic and alkaline pH decreased the association of the peptides which was, furthermore, completely abolished by 500 mM NaCl, confirming that charged residues are critical for heterodimerization. A commercially obtained recombinant Jun protein competed as efficiently as the Jun leucine zipper peptide for binding to the Fos peptide, confirming the feasibility of using the two leucine zipper peptides to study the interactions between the two transcription factors. We also injected leucine zipper peptides individually into *Xenopus* oocytes to study whether they would interfere with the activity of the Fos/Jun heterodimer *in vivo*. Both peptides blocked selectively insulin-mediated oocyte maturation with an IC_{50} in the range of 15 ng per oocyte. In conclusion, the scintillation proximity assay described here may be used to investigate protein–protein interactions mediated by leucine zipper structures and to identify compounds that inhibit leucine zipper association.

Many types of transcription factors bind to DNA as dimers (MacGregor et al., 1990; Hu et al., 1990). Transactivation-competent heterodimeric complexes are required to regulate the expression of many genes and to induce transformation (Shuermann et al., 1989; Neuberg et al., 1991). The best example of cooperating factors is given by the Jun and Fos protooncogenes, the expression of which is one of the earliest nuclear responses to a wide variety of growth and differentiation factors [for reviews, see Ransone and Verma (1990)]. Activation of an AP1 binding site (the consensus core DNA sequence TGACTCA), which is the target of the Fos and Jun protooncogene products (Distel et al., 1987; Franza et al., 1988; Rauscher et al., 1988), has been correlated with an increased transformation potential of the ras oncogenes (Sassone-Corsi et al., 1989). It was recently shown that mutated Jun or Fos proteins deficient in DNA binding activity (Okuno et al., 1991) lacking an activation domain (Lloyd et al., 1991; Yen et al., 1991) or a functional leucine zipper domain (Wick et al., 1992) were able to competitively inhibit Fos/Jun transcriptional activity. A similar mechanism has been evoked for Δ fos B, a truncated product of the Fos B gene which can also regulate Fos and Jun. Δ Fos B retains the leucine repeat and basic region of Fos, but lacks an activation domain (Nakabeppu & Nathans, 1991).

Some of these mutant proteins were also found to repress transformation induced by a ras oncogene and to promote partial reversion of the transformed phenotype without having a significant effect on normal cell proliferation (Lloyd et al., 1991; Wick et al., 1992). Disruption of both copies of the human jun (c-jun) or fos (c-fos) genes by homologous recombination in a mouse embryonic stem line was shown to have no apparent effect on the viability, the growth rate, or the *in vitro* differentiation potential of these cells (Field et al., 1992; Hilberg et al., 1992). Thus, molecules capable of inhibiting the function of such proteins by blocking their ability to form heterodimers could represent new therapeutic agents. However, it is clear that knowledge of the structure of the site of heterodimerization would be an imporant asset in the search for active molecules.

The dimerization of Jun and Fos is mediated by a structure known as the leucine zipper domain (Landschultz et al., 1988), which serves to juxtapose helical regions of each protein. These regions are rich in basic amino acids and form a bipartite DNA binding domain (Kourazides & Ziff, 1988). The Fos protein by itself does not dimerize well and therefore cannot bind DNA, whereas the Jun protein can form homodimers, which can then bind to the Fos/Jun DNA target, constituted by the TPA-responsive element. However, the Jun homodimer is less stable than the Jun/Fos heterodimer, the predominant species in many cell types (Franza et al., 1988). To study the mechanism of specificity in Jun/Fos heterodimer formation, we synthesized peptides corresponding to the leucine zipper domains of the two proteins. These Jun and Fos leucine zipper peptides preferentially formed a heterodimer, suggesting that these sequences are sufficient to mediate preferential heterodimer formation of the intact Fos and Jun proteins, as has been previously described (O'Shea et al., 1989). Several of the physicochemical properties of the interaction between the Fos and Jun leucine zipper peptides, including binding affinities, were examined in the present study by the use of

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a scintillation proximity assay (SPA).[1] These peptides were also found to interfere with an insulin signal transduction pathway upon injection in *Xenopus laevis* oocytes.

EXPERIMENTAL PROCEDURES

Synthesis, Purification, and Characterization of Peptides. Peptides (Figure 1) were synthesized in the solid phase on a [(hydroxymethyl)phenoxymethyl]polystyrene resin by using FMOC (9-fluorenylmethoxycarbonyl) chemistry on a small scale, on an Applied Biosystems Model 431A peptide synthesizer. All the solvents and reagents used for synthesis were obtained from Applied Biosystems, except Fmoc-β-alanine, which was obtained from Novabiochem. All the peptides were synthesized with an extra C-terminal tyrosine residue for the purpose of ^{125}I labeling. For the synthesis of N-acetylated peptides, the acetyl moiety was introduced directly on the synthesizer by using a standard coupling procedure and acetic acid instead of the protected amino acid. The N-biotinylated peptide was synthesized by a procedure derived from that of Hoffman et al. (1984). Briefly, unbiotinylated peptide was synthesized as described above, the terminal Fmoc protecting group was removed, and the peptide linked to resin was dried. Biotin (2 mmol, Aldrich) was mixed with an equimolar quantity of carbonyldiimidazole (Aldrich) in 10 mL of dimethylformamide (Prolabo) for 3 h at 80 °C. After being cooled to room temperature, 2 mmol of hydroxysuccinimide (Aldrich) was added, and the mixture was left to react for 3 h. The peptide linked to the resin was then added and left, with gentle stirring, to react overnight.

Protecting groups were removed from the peptides, and the peptides were cleaved off the resin in a mixture of trifluoroacetic acid (TFA)/phenol/ethanedithiol/thioanisole/water (40:3:1:2:2, v/v; King et al., 1990) for 1.5 h and then precipitated by the addition of *tert*-butyl methyl ether. After being dried under vacuum, crude peptides were dissolved in a mixture of water/acetic acid/acetonitrile (8:1:1, v/v). Crude peptides were analyzed by high-performance liquid chromatography (HPLC) on a BioRad RSL C_{18} 100-Å column (4.6 × 150 mm) eluted by a 30-min linear gradient of 0–80% solvent B (acetonitrile containing 0.07% TFA v/v) in solvent A (water containing 0.07% TFA v/v). Peptides were purified by preparative HPLC on a BioRad RSL C_{18} 100-Å column (10 × 150 mm), eluted in the same solvent system, and a preparative gradient was set up for the analytical separation. The purity of each peptide was checked by using the analytical HPLC method described above; all peptides were 95% pure. All peptides were analyzed by liquid secondary ion mass spectrometry (LSIMS) on an Autospec mass spectrometer (VG Analytical, Manchester, U.K.). Measured molecular weights were close to the calculated values (difference <0.3 atomic mass unit).

Thermal unfolding was studied by circular dichroism (CD), with a Jobin Yvon Mark V autodichrograph in order to control the specificity of the association between the Fos and Jun peptides. We compared T_m values (temperature at which the folded fraction is equal to the unfolded one) for the Jun(z) and the Fos(z) (Table I) homodimers with the T_m value for the heterodimer. CD spectra were recorded in a 2-mm pathlength cell at a total peptide concentration of 50 μM for all the experiments. Peptides were prepared in 150 mM NaCl/10 mM sodium phosphate buffer (pH 7.5). Thermal curves were determined by monitoring the CD signal between 200 and 250 nm, as a function of temperature, which ranged between 3 and 70 °C. Each curve represents an average of three measured spectra: T_m values were determined by taking the first derivative of the CD signal at the different temperatures. The effect of detergent on heterodimer thermal stability was measured by comparing T_m values for the heterodimer obtained under our experimental conditions with or without 0.1% Triton X-100 in the buffer.

Fos(z) Labeling and ^{125}I-Fos Purification. Fos(z) peptide was labeled by the chloramine-T method (Greenwood et al., 1963; Hunter & Greenwood, 1962). Briefly, the oxidation reaction was initiated by adding 0.6 mg of the Fos(z) peptide in 175 μL of 0.25 M sodium phosphate, pH 7.5, and 0.5 mCi (5 μL) of Na^{125}I (Amersham) to 28 μL of chloramine-T at a concentration of 1 mg/mL. The reaction was continued for 45 s at room temperature and then stopped by adding 100 μL of a 1 mg/mL sodium metabisulfite solution. ^{125}I-Fos(z) was separated from the unlabeled Fos(z) by reverse-phase HPLC on a Gilson Model 802 apparatus with an analytical Vydac C_{18}, 300-Å column at room temperature. We used a linear acetonitrile/H_2O gradient with segments of 0.5% acetonitrile increase per minute in the presence of 0.1% TFA.

Fos/Jun Scintillation Proximity Assay. Fluorophor-containing poly(vinyltoluene) (PVT) beads, 2–8 μm in diameter, coated with streptavidin, were purchased from Amersham Corp. We used ^{125}I-Fos(z) as the free, labeled ligand and biotinylated Jun(z) immobilized on the PVT beads. In the assay, Auger electrons emitted by ^{125}I-Fos(z) excite the fluorophor in the beads only when they are in proximity, that is, only when ^{125}I-Fos(z) is bound to biotinylated Jun(z) [biot-Jun(z)], which is linked to the beads. Auger electrons resulting from the decay of unbound ^{125}I-Fos(z) are dissipated into the aqueous medium and cannot induce excitation of the fluorescent beads. The luminescence generated by the binding of ^{125}I-Fos(z) to biot-Jun(z) was measured by using a Pharmacia 1205 Betaplate counter with the window set for full range detection of ^{3}H. The assay buffer was phosphate-buffered saline (10 mM sodium phosphate/150 mM NaCl, pH 7.2) supplemented with 0.1% Triton X-100.

(*A*) *Saturation binding kinetics* were studied by adding increasing concentrations of ^{125}I-Fos(z) (0.1 nM–5 μM final, 0.02 μCi/μg) to biot-Jun(z) (10 nM) in 230 μL of assay buffer in microplates and incubating the reaction mixture for 10 min at room temperature. Dimers, which were stable for at least 60 min, were then captured by adding 20 μL (400 μg) of PVT beads to the assay buffer. The microplates were then sealed, and the light emission was recorded in a liquid scintillation spectrophotometer as described above. Specific binding was defined as the difference between the total and the nonspecific binding, which was determined with a 100-fold excess of unlabeled Fos(z). Assays were performed in triplicate in at least three independent experiments, and the data were expressed as mean ± SEM. The equilibrium dissociation constant, K_d, was calculated by linear regression analysis of the Scatchard plot (Scatchard, 1949).

The influence of pH and salts on the formation of Fos(z)/Fos(z) homodimers and Fos(z)/Jun(z) heterodimers was studied as follows: we measured the specific binding of ^{125}I-Fos(z) (0.1 nM, 373 μCi/μg) to biot-Jun(z) (1 nM) or to biot-Fos(z) (1 nM) in the assay buffer containing 0.15 M NaCl at a pH ranging from 3 to 10 or in 10 mM sodium phosphate buffer, pH 7.2, at various NaCl concentrations (50, 150, 250, and 500 mM); 0.1% Triton X-100 was systematically added to the assay buffer.

[1] Abbreviations: SPA, scintillation proximity assay; FMOC, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LSIMS, liquid secondary ion mass spectrometry; CD, circular dichroism; PVT, poly(vinyltoluene); PBS, phosphate-buffered saline; SEM, standard error of the mean; MBS, modified Barth's solution; GVBD, germinal vesicle breakdown.

(*B*) *Competitive binding experiments* were performed as follows: biot-Jun(z) (10 nM) and ^{125}I-Fos(z) (0.1 nM, 373 μCi/μg), to which had been added the different nonradioactive synthetic peptides (Figure 1), at concentrations ranging from 0.1 nM to 10 μM, or recombinant Jun protein (Promega) or HIV integrase (American Biotechnologies), at concentrations ranging from 1 nM to 0.5 μM, were incubated for 10 min at room temperature in 225 μL of assay buffer. Twenty-five microliters (250 μg) of PVT/streptavidin beads was then added to the buffer. In these experiments, nonspecific binding was determined by incubating ^{125}I-Fos(z) (0.1 nM, 373 μCi/μg) with PVT/streptavidin beads in assay buffer and omitting the biot-Jun(z). This method of evaluating nonspecific binding was found in control experiments to be equivalent to that found by isotopic dilution with a 100-fold excess of unlabeled Fos(z). Assays were done in duplicate in at least three independent experiments. IC_{50} values (the concentration of the antagonist that inhibits 50% of specific ^{125}I-Fos binding) were calculated by using a nonlinear regression analysis program (Enzfitter, Elsevier-Biosoft, 1987).

Microinjection and Maturation of Oocytes. Adult *Xenopus laevis* females were obtained from CRBR of CNRS (Montpellier, France) and maintained as described (Le Gascogne et al., 1984). Lobes of the ovaries were surgically removed and freed of follicle cells with collagenase (Sigma type 1A) treatment for 2 h in Ca^{2+}-free modified Barth's solution (MBS). Occytes were mtained in MBS at 18 °C for 16–20 h before injection. Healthy oocytes were visually selected and injected by using an automatic pressure generator.

The Fos(z) and Jun(z) peptides, diluted in PBS buffer containing 0.1 mg/mL BSA, were used at the following concentrations: 2.5, 1.25, 0.625, and 0.25 mg/mL. Each of the peptide solutions, as a volume of 40 nL, was microinjected into the cytoplasm of 1 oocyte (10 oocytes were used for each peptide solution). A 10-nL volume of each peptide solution was injected into the nucleus of each of another group of 10 oocytes (the same final amount of each peptide per oocyte, but in a smaller volume than that used for the cytoplasm). The microinjected oocytes were incubated in 3 mL of MBS, with or without either 1 μM insulin or 1 μM progesterone, for 18 h at 18 °C.

Oocytes were examined microscopically for maturation, indicated by the appearance of a white spot in the pigmented animal pole. This is a sign of germinal vesicle breakdown (GVBD), which was analyzed by dissection of the oocytes preincubated in 5% trichloroacetic acid (Maller & Knoontz, 1981).

RESULTS

Characterization of Leucine Zipper Peptides. All the peptides synthesized are presented in Figure 1. The longest peptides, Fos(z) and Jun(z), started five residues before the first leucine at the amino end and included six residues after the fifth leucine at the carboxy end. An extra terminal tyrosine was added to the carboxy terminus of each peptide so that the peptides could be labeled with ^{125}I. Truncated versions of these leucine zipper domains, Fos(z)COOH, Fos(z)NH_2, Jun(z)COOH, and Jun(z)NH_2, were also produced. All peptides displayed a final purity greater than 95% when tested on reverse-phase HPLC and all were characterized by mass spectrometry (see Experimental Procedures).

Circular dichroism measurements were made to control the relative thermal stability of the Fos(z) and Jun(z) homodimers and heterodimers. The results, presented in Table I, show that the Fos(z) peptide homodimer was less stable

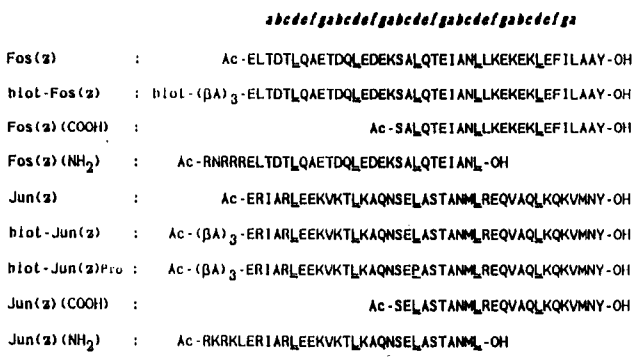

FIGURE 1: Amino acid sequences of the peptides used in the assay. Amino acids are given in the one-letter code (IUPAC). Abbreviations: Fos(z) = [*N*-acetyl, Tyr200] human c-Fos 160–200; biot-Fos(z) = [*N*-(biotinyl-tri-β-alanyl), Tyr200] human c-Fos 160–200; Fos(z)COOH = [*N*-acetyl, Tyr200] human c-Fos 177–200; Fos(z)NH_2 = [*N*-acetyl] human c-Fos 155–186; Jun(z) = [*N*-acetyl, Tyr315] human c-jun 275–315; biot-jun(z) = [*N*-(biotinyl-tri-β-alanyl), Tyr315] human c-Jun 275–315; Jun(z)COOH = [*N*-acetyl, Tyr315] human c-Jun 292–315; Jun(z)NH_2 = [*N*-acetyl] human c-Jun 270–309.

Table I: T_m for Homodimers and Heterodimers[a]

peptides	T_m (°C)	peptides	T_m (°C)
Fos(z)–Fos(z)	13	Jun(z)–Fos(z)	48
Jun(z)–Jun(z)	29	Jun(z)–Fos(z)[b]	48

[a] Conditions: peptide concentration of 50 mM in 150 mM NaCl/10 mM sodium phosphate (pH 7.5). [b] 0.1% Triton X-100 added to buffer. No significant CD signal was obtained for Jun(z)COOH, Jun(z)NH_2, Fos(z)COOH, or Fos(z)NH_2.

than either the Jun(z) homodimer or the Fos(z)–Jun(z) heterodimer, as expected from published dimerization studies of Fos and Jun proteins (Schuermann et al., 1991). Similar circular dichroism studies of disulfide peptide dimers have been reported by O'Shea et al. (1992). Each of their T_m values was higher than those we obtained, probably due to the presence of engineered disulfide bridges in their peptides, but relative to each other, their values were comparable to ours. The addition of 0.1% Triton X-100 to the SPA buffer did not modify the Fos(z)–Jun(z) T_m value (Table I). We sought a suitable linker to be inserted between the biotin residue and the amino terminus of the Jun(z) peptide to facilitate its access to the beads and to avoid problems of steric hindrance, which could cause peptide dimers to dissociate from the beads. A stronger signal was obtained for the binding of ^{125}I-Fos(z) to biot-Jun(z) on the PVT beads by inserting a tri-β-alanine spacer between biotin and the Jun(z) peptide (data not shown). Other spacers (diglycyl, 5-aminohexanoyl, 8-aminooctanoyl, 12-aminododecanoyl) were found to be less efficient. We tried to make another spacer by grafting two glycines and a cysteine residue onto the NH_2 terminus of the Jun(z) peptide, as described by O'Shea et al. (1989), in order to link it onto NH_2-derivatized beads. However, in our hands, this experiment failed because of uncontrolled oxidation of the cysteine residue (data not shown).

Characterization of Fos/Jun Association by the SPA. By using the SPA, the binding of iodinated Fos(z) to biot-Jun(z) could be measured without physical removal of the unbound peptides (see Experimental Procedures). Binding of ^{125}I-Fos(z) to biot-Jun(z) was rapid and saturable (Figure 2A). Ten min incubation was sufficient for the binding to reach equilibrium, and the signal observed was stable for over 2.5 h. The specificity of this binding was assayed by the use of a mutant peptide, biot-Jun(z)Pro (Figure 1), in which a leucine residue was substituted by a proline residue, a substitution that is known to disrupt the helical structure of Jun(z). When

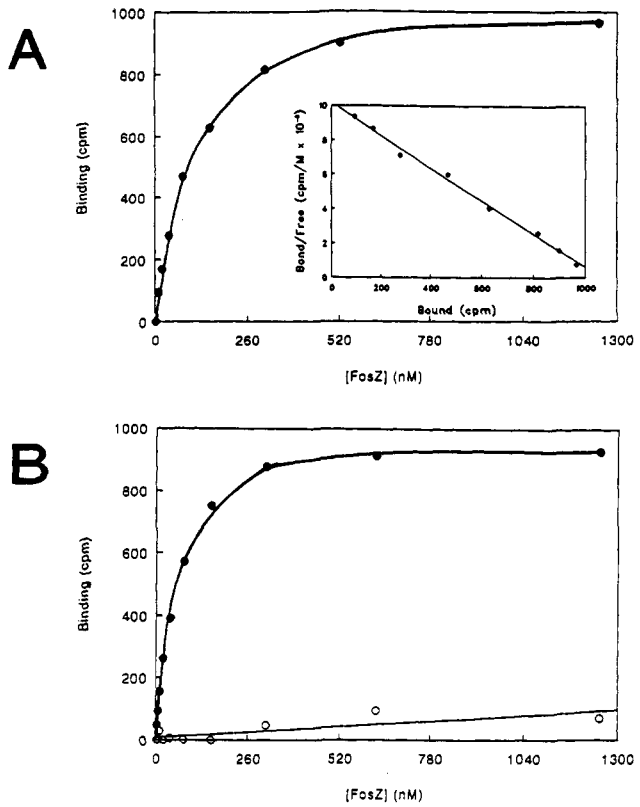

FIGURE 2: Representative experiment of saturation analysis of ^{125}I-Fos(z) binding to biot-Jun(z). Binding was determined by the scintillation proximity assay described in the text. (A) The Scatchard plot analysis revealed one class of binding sites with K_d = 110 ± 12 nM. Each point represents the average of duplicate samples (number of experiments = 3). (B) Comparison of the binding of ^{125}I-Fos(z) to biot-Jun(z) (●) and to biot-Jun(z)Pro (○). Each point represents the average of two determinations.

this mutant peptide was used, no specific binding was observed (Figure 2B).

Scatchard analysis (Scatchard, 1949) of Fos(z)biot-Jun(z) dimerization yielded an average dissociation constant (K_d) of 110 ± 12 nM (Figure 2A). The reverse experiment, binding of ^{125}I-Jun(z) to biot-Fos(z), could not be done because we were not able to label the Jun(z) peptide in sufficient yield (data not shown).

We had expected the Jun(z) and Fos(z) peptides to both homodimerize and heterodimerize, but because we used two distinct labels (biotin and iodine), only the heterodimerized species could be detected. Fos(z) was a 5-fold better competitor than Jun(z) for binding to biot-Jun(z) [IC_{50} for Fos(z), 87 ± 4.5 nM; IC_{50} for Jun(z), 476 ± 15 nM] (Figure 3A,B). These results confirm those of previous studies showing that these peptides could compete with the DNA binding activity of a hybrid lexA/Jun repressor (Schmidt-Dörr et al., 1991). Jun(z) may have homodimerized in the assay buffer and, in this form, would not be able to compete for binding; this may be why less competition was observed with this peptide than with Fos(z). The truncated leucine zipper peptides Jun(z)COOH, Jun(z)NH_2, Fos(z)COOH, and Fos(z)NH_2 (Figure 1) could not compete with Fos(z) at concentrations up to 10 μM for the binding to biot-Jun(z) (Figure 3A,B). Circular dichroism studies performed with these peptides showed that they were not folded; furthermore, when their concentration was raised, no evidence of homo- or heterodimerization was observed (data not shown).

The Jun protein in its native form efficiently competed with biot-Jun(z) for binding to ^{125}I-Fos(z) with an estimated IC_{50} of 137 ± 20 nM (Figure 4). Recombinant HIV integrase,

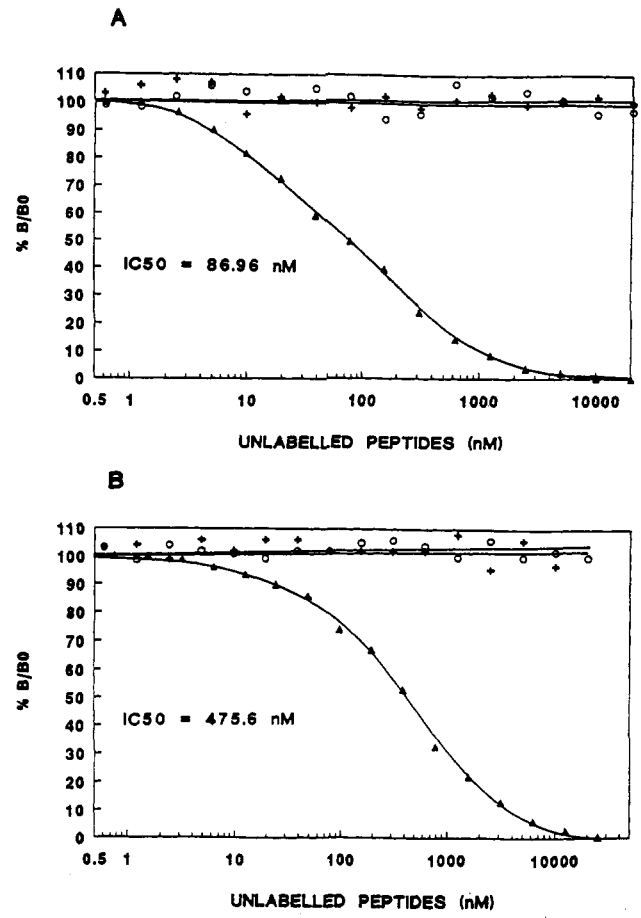

FIGURE 3: Competition experiments. The value for each concentration was determined in duplicate. The data represent the mean ± SEM of three separate experiments. (A) Competition of nonradioactive Fos(z) (▲), Fos(z)COOH (○), or Fos(z)NH_2 (+) with ^{125}I-Fos in the cross-linking to biot-Jun(z). (B) Competition of nonradioactive Jun(z) (▲), Jun(z)COOH (○), or Jun(z)NH_2 (+) in the association of ^{125}I-Fos to biot-Jun(z).

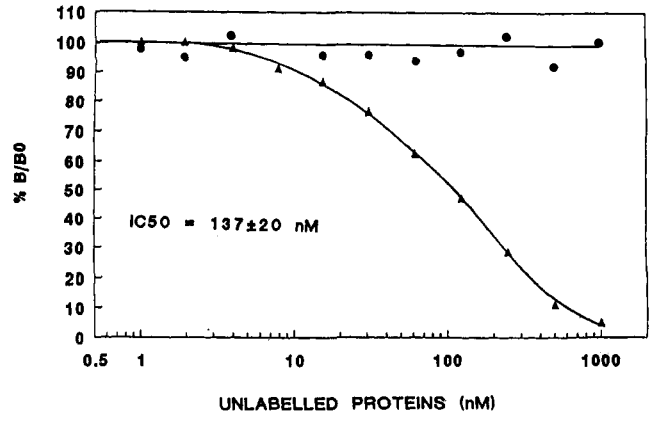

FIGURE 4: Competition of nonradioactive recombinant Jun protein (▲) or recombinant HIV integrase (●) in the association of ^{125}I-Fos to biot-Jun(z). The value for each concentration was determined in duplicate. The IC_{50} value determined for the integrase represents the mean ± SEM of three separate experiments.

which also bears a leucine zipper domain (Lin & Grandgenett, 1991) and which has a size close to that of the Jun protein, did not compete (Figure 4). These results showed that Fos(z) was able to specifically recognize the leucine zipper domain within the intact Jun protein in vitro.

The effect of pH and ionic strength on homo- and heterodimerization was evaluated at a saturation concentration of ^{125}I-Fos(z). Heterodimer formation was optimal at pH 6 and decreased by about 90% at pH 4 (Figure 5A). Het-

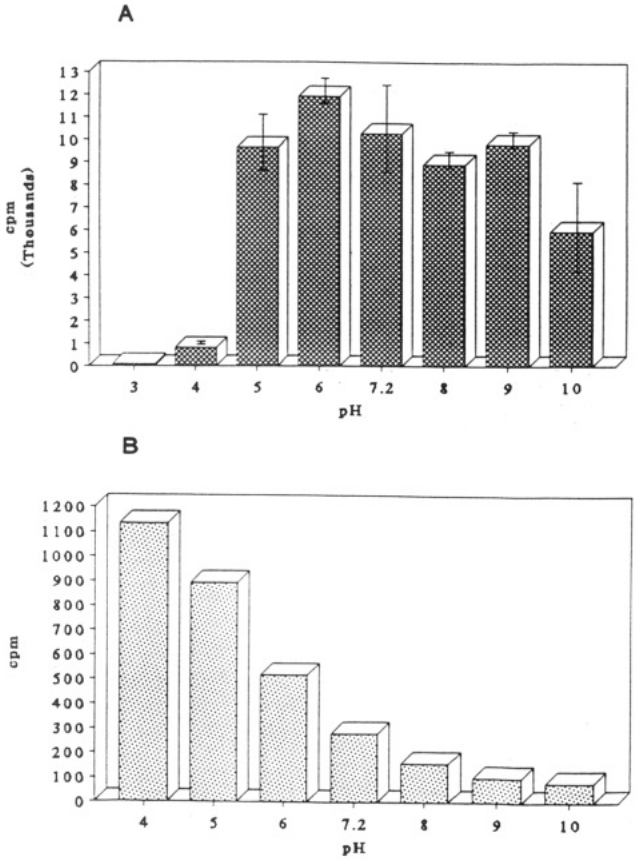

FIGURE 5: pH dependence of the dimerization of the leucine zipper peptides, determined by SPA. The specific binding of ^{125}I-Fos(z) (0.1 nM) to biot-Jun(z) (1 nM) (A) or to biot-Fos(Z) (1 nM) (B) was measured in assay buffer pH ranging from 3 to 10. Results for the heterodimers represent the mean ± SEM of three separate experiments. In the case of Fos homodimerization, each value is the mean of duplicate determinations of a representative experiment.

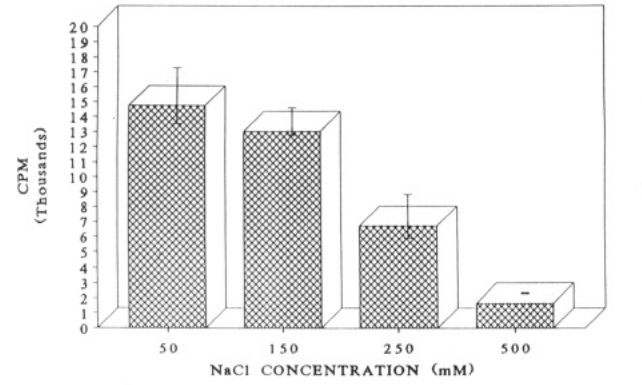

FIGURE 6: Influence of salt concentration on the dimerization of the leucine zipper peptides, determined by SPA. The specific binding of ^{125}I-Fos(z) (0.1 nM) to biot-Jun(z) (1 nM) in 10 mM sodium phosphate buffer, pH 7.2, at NaCl concentrations of 50, 150, 250, and 500 mM was measured. The results represent the mean ± SEM of three separate experiments.

erodimerization was less perturbed at basic pH, as it significantly decreased (by 50%) only at pH 10. In contrast, lowering the pH increased the Fos(z)/biot-Fos(z) association (Figure 5B): no significant homodimerization of this peptide was detected at neutral pH to be contrasted with the signal observed at pH 4. The Fos(z)/biot-Jun(z) association was not affected by varying the ionic strength of the assay buffer between 50 and 150 mM NaCl, but it decreased by about 50% when the NaCl concentration was raised from 150 to 250 mM (Figure 6). This heterodimerization was abolished by 50 mM NaCl.

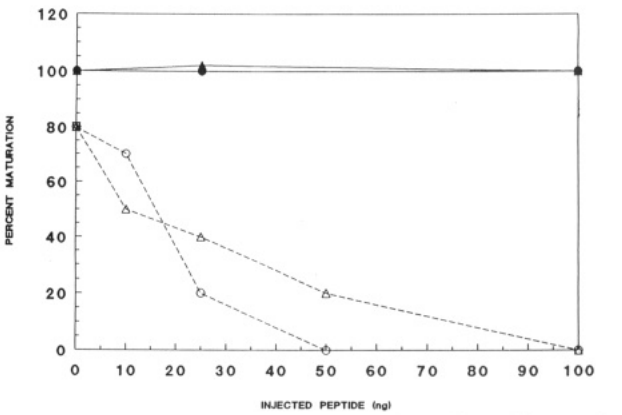

FIGURE 7: In vivo effects of the Fos(z) and Jun(z) peptides on the maturation of *Xenopus* oocytes induced by insulin or progesterone. Both insulin and progesterone were present at concentrations of 1 μM. The following data are shown: progesterone + Fos(z) (●); progesterone + Jun(z) (▲); insulin + Fos(z) (○); insulin + Jun(z) (△).

Effect of Fos(z) and Jun(z) on Xenopus Oocyte Maturation. The stability and biological activity of the Fos(z) and Jun(z) peptides were assessed by injecting them into *Xenopus* oocytes. Over 50% of the full-length peptides could be recovered 1 h after injection (data not shown). The GVBD induced by 1 μM insulin was suppressed in a concentration-dependent manner by either the Fos(z) or the Jun(z) peptide, with an IC_{50} of about 15 ng of peptide per oocyte (corresponding to a 3 μM peptide concentration) (Figure 7). However, neither peptide inhibited the progesterone-induced GVBD, suggesting that this pathway, in order to function, does not require the Fos/Jun complex. Interestingly, injection into the nucleus of the same amount of Jun(z) and Fos(z) peptides did not block GVBD induced by insulin (data not shown), indicating that the Jun and Fos leucine zipper peptides are effective only when injected into the cytosol.

DISCUSSION

We describe in this report a new approach to the study of protein association, based on SPA technology. We focused on the leucine zipper domains of the oncoproteins Fos and Jun, the heterodimerization of which is essential to their activity as transcription factors. Short peptides containing the leucine zipper of these proteins were constructed and found to heterodimerize under the conditions of the assay, giving rise to a specific easily detected signal.

The value we obtained for the dissociation constant of the Jun(z)/Fos(z) heterodimer was 110 ± 12 nm (in PBS + 0.1% Triton X-100). Certain leucine zipper domains can, however, dimerize with much higher affinities. For example, a dissociation constant of 4 pM has been reported for the homodimerization of a model leucine zipper peptide (O'Neil et al., 1990). That the value we obtained is low may reflect a certain flexiblity inherent in inducible systems involving several membered protein families, such as Fos and Jun [possible dimerization between each member of the Fos and Jun family and cross-talk with the CREB/ATF transcription factors (Bush & Sassone-Corsi, 1990)].

Several features of the heterodimerization we observed can be extrapolated to the dimerization of the native proteins Fos and Jun, because the IC_{50} of Jun(z) is similar to that of Jun. However, we cannot exclude the fact that residues outside of the two leucine zipper domains may be involved in the heterodimerization process as it has been suggested (Cohen & Curran, 1990).

The Jun(z)NH_2 and Fos(z)COOH peptides do not compete for the association of the full-length Fos and Jun leucine zipper

peptides. Although Jun(z)NH_2 can theoretically provide two-thirds of the interacting residues present in Jun(z) (positions a, d, e, and g; Figure 1), at the highest concentration used in our competition studies (10 μM) Jun(z)NH_2 may not have been present in sufficient quantity to significantly inhibit dimerization [IC_{50} of the Jun(z) peptide = 476 ± 15 nM]. However, it is also possible that these shorter peptides cannot fold into the proper conformation required for coiled-coil formation. Indeed, circular dichroism studies have not revealed any helical structure within these properties (data not shown). Jun(z)NH_2 does contain three full heptad repeats, which has recently been suggested to be the minimal length necessary for coiled-coil formation (Bracco et al., unpublished results); however, the carboxy terminus of this peptide may not be entirely functional.

We found that heterodimer formation varies as a function of the pH. These results correlate with those of recent studies in which the thermal stability of the Fos and Jun Homo- and heterodimers was shown to be pH-dependent (O'Shea et al., 1992). Electrostatic effects favor heterodimer formation in the Fos/Jun dimer at neutral pH. Lowering the pH increases the homodimer association of the Fos(z) peptide (Figure 4B). These results agree with data suggesting that the peptide homodimers are destabilized at neutral pH by residues of like charge: the Fos homodimer by acidic residues and the Jun homodimer, to a lesser extent, by basic residues. Perturbation of heterodimer formation by lowering the pH could be the result of an increase in Fos homodimer formation. On the other hand, Jun(z) and Fos(z) peptides do not associate in the presence of NaCl at high concentrations, confirming the results of several mutagenesis experiments targeting various positively and negatively charged residues (Schuermann et al., 1991). Interestingly, O'Shea et al. did not observe any salt-associated effects in their circular dichroism experiments, probably due to the limitations of the technique. The SPA, we think, is a more sensitive tool for detecting small differences in the formation of complexes between peptides at submicromolar concentrations.

That the leucine zipper peptides Fos(z) and Jun(z) could form specific dimers with the native Jun protein may be why the insulin-induced GVBD, an index of oocyte maturation, was inhibited when these peptides were injected into the cytoplasm. Such peptide/protein complexes may be either sequestered in the cytoplasm or translocated into the nucleus in a form that cannot associate with an AP1 site. The lack of inhibition when these peptides are injected into the nucleus suggests that either the Fos or the Jun protein leucine zipper domains are less accessible than they are in the cytoplasm or that these peptides are more extensively degraded.

Circular dichroism is the method usually used to study leucine zipper peptide association. This technique measures changes in secondary structure, yielding only indirect data for a number of parameters relevant to the study of protein dimers. The SPA described here is a direct, rapid, and easy technique to use. It could be readily scaled-up to serve as a primary screening to detect, for example, molecules that inhibit the dimerization of leucine zipper domains. Furthermore, the SPA is useful not only for studying Fos/Jun association but also for examining specific homo- and heterodimeric interactions within other transcription factors and, more broadly, other protein–protein interactions involved in many biological and pathological processes.

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