Biochemical and functional analysis of highly phosphorylated forms of c-Jun protein

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Abstract We report here that, upon UV irradiation or growth stimulation, endogenous c-Jun (40 kDa) in chicken embryo fibroblasts (CEF) is converted into several forms with apparently higher molecular weights in SDS-polyacrylamide gel electrophoresis (45, 44, 42 kDa). Two of the bands (44 and 45 kDa) were transient after growth stimulation, but were much more persistent after UV irradiation. In both cases, the drastic mobility shifts were accompanied with the activation of endogenous JNK activity but not of MAPK activity, and the bands were shown to represent different phosphorylation states of c-Jun rather than ubiquitinated c-Jun. Biochemical analysis indicated that phosphorylation at Ser^{63} and Ser^{73} was not sufficient to produce these drastic mobility shifts, which additionally required phosphorylation at Thr^{91} and Thr^{93}. Substitution of both Ser^{63} and Ser^{73} with either Ala or Asp had no significant effect on the transforming activity of c-Jun, but the mutants failed to show drastic mobility shifts even after UV irradiation. These results indicate that Ser^{63} and Ser^{73} are essential for the drastic mobility shifts and further suggest that the highly phosphorylated forms of c-Jun are not directly involved in cellular transformation.

Key words: c-Jun; c-Jun N-terminal kinase; Phosphorylation; Cellular transformation; Ultraviolet irradiation

1. Introduction

A significant body of evidence indicates that both transient and constitutive stimulation of (or in) cells results in transduction of signal to transcriptional factor complexes such as AP-1 (composed of Fos and Jun family proteins). The process is thought to involve covalent modification or changes in the rate of de novo synthesis of constituent proteins, resulting in modulation of the composition of the complexes [1–3]. One component of AP-1, c-Jun protein [4], has attracted particular interest because of its broad and pivotal range of biological functions in cellular growth, differentiation, transformation and apoptosis. For example, high-level expression of c-Jun alone is sufficient to induce cellular transformation in chicken embryo fibroblasts (CEF) [5–7] or established rodent fibroblasts [8]. Constitutive expression of such oncogenes as *src, ras* and *raf* in CEF, was shown to cause the elevation of endogenous AP-1 activity via the elevation of c-Jun and Fra-2 expression [9]. Since dominant negative mutants of Fos or Jun family proteins efficiently suppress transformation induced by these oncogenes, AP-1 activation is essential for these cellular transformations [9,10].

The N-terminal region of c-Jun is phosphorylated by c-Jun N-terminal kinase (JNK) [11–13] and the resultant phosphorylated c-Jun has been suggested to play important roles in response to stress or, in some cases, in the maintenance of cellular transformation [14,15]. c-Jun is also efficiently ubiquitinated both in vitro and in a novel in vivo assay that utilizes molecularly-tagged ubiquitin [16].

During protein analysis of AP-1 components in CEF, we have encountered drastic mobility shifts of c-Jun protein upon either UV irradiation or growth stimulation. We have therefore analyzed the molecular events involved in these mobility shifts and examined the possible biological function of these modified forms of c-Jun, especially from the standpoint of cellular transformation.

2. Materials and methods

2.1. Cell culture and preparation of cell lysates

CEF were kept in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS), 10% triptose phosphate broth and 1% DMSO [10]. For starvation, CEF were kept in MEM supplemented with 0.2% CS for 24 h as described previously and growth-stimulated by adding CS to a final concentration of 15% to the culture medium [17,18]. For UV irradiation, CEF were kept in MEM without phenol red, but supplemented with 5% CS, and irradiated using a 254-nm germicidal lamp (UVP, Inc., Upland, CA, USA) at the dose of 20 J/m^2 [18]. CEF were pulse-labeled with [^{35}S]L-methionine (600 μCi per 35-mm dish) for 20 min or with [^{32}P]H_3PO_4 (500 μCi per 35-mm dish) for 2 h as described previously [19]. For immunoprecipitation analysis, cell lysates were prepared under denaturing conditions in the presence of phosphatase inhibitors (5 mM Na_3VO_4 and 10 mM NaF) [18,19]. The cell lysate for in-gel kinase assay and for Western blotting analysis was prepared as follows: cells were washed twice with PBS, disrupted by adding the sample buffer (61.2 mM Tris-HCl, pH 6.8, 1.96% SDS, 9.8% glycerol and 2.45% β-mercaptoethanol) and boiled for 15 min. Protein amounts were determined by using a Bio-Rad protein assay kit.

2.2. Construction of plasmids

*Bgl*II-*Bam*HI fragments (1.0 kb) encoding the wild-type or mutant c-Jun (rat) fused to a 6× histidine tag at the N-terminus were isolated from pCB-wtJ, pCB-SSAA and pCB-SSDD (a kind gift from T. Curran) [20,21]. They were inserted into the unique *Bgl*II site of pDS3 [22] to generate pDS3-wtJ, pDS3-SSAA and pDS3-SSDD, respectively. The 1.3-kb *Hin*cII fragment encoding JNK1 (a kind gift from R.J.

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Abbreviations: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; CIP, calf intestine alkaline phosphatase; CEF, chicken embryo fibroblasts

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Davis) [14] was inserted into the unique *Sma*I site of a chimeric vector, pSK118, which was generated by ligating the 1.8-kb *Sca*I-*Sma*I fragment of pBluescript (SK+) and the 1.4-kb *Sca*I-*Sma*I fragment of pUC118. The resultant plasmid was digested with *Bam*HI, and the fragment encoding JNK1 was inserted into the unique *Bgl*II site of pDS3 to generate pDS3-JNK1.

2.3. Virus vector preparation

For the production of recombinant viruses, pDS3-wtJ, pDS3-SSAA, pDS3-SSDD, pJH-1 (encoding human c-Jun) [6] and pDS3-JNK1 were each completely digested with *Sal*I and ligated with the *Sal*I digest of pREP(A) to form the structure of a replication-competent provirus (subgroup A). Ligated DNAs (2 μg) were transfected into CEF as described previously [22], and replication-competent virus stocks were collected from the culture 5 or 6 days after transfection.

2.4. Protein analysis

The ^{35}S- or ^{32}P-labeled lysates were immunoprecipitated with anti-c-Jun (#14) antiserum which is non-crossreactive to other Jun family proteins, as described previously [19]. For Western blotting, whole cell extracts (20 μg) were separated by electrophoresis on SDS-10% polyacrylamide gel, immunoblotted with affinity-purified anti-c-Jun (#10) antiserum [6] and visualized by use of the ECL Western blotting detection system (Amersham). In-gel kinase assays for JNK and MAPK were performed as described previously using the c-Jun N-terminal region produced by pQE30 (c-JunCD) (40 μg/ml) or MBP (myelin basic protein, 0.5 mg/ml) as the substrates [11,18,23].

For dephosphorylation analysis, the immunoprecipitates were solubilized by boiling for 2 min in buffer I (50 mM Tris-HCl, pH 8.0, 2 mM PMSF, 0.05 mg/ml antipain, 0.05 mg/ml leupeptin, 0.5% Trasylol, 0.3% SDS and 5 mM DTT), and then diluted with 3 volumes of buffer II (1.3% NP-40, 1.3% deoxycholate-Na and 200 mM NaCl) before being treated with calf intestinal alkaline phosphatase (CIP) [24].

For tryptic mapping, ^{32}P-labeled c-Jun protein bands separated on SDS-10 polyacrylamide gel were excised from the gels, subjected to trypsin digestion and applied to thin layer cellulose plates for two-dimensional peptide mapping as described previously [25].

3. Results and discussion

3.1. Transient and prolonged induction of drastic mobility shifts of c-Jun

For the analysis of AP-1 components synthesized after UV irradiation, logarithmically growing CEF were pulse-labeled before and after UV irradiation and disrupted to prepare cell lysates under denaturing conditions at the time indicated (Fig. 1, upper panel). Similarly, cell lysates were prepared from pulse-labeled cultures before and after growth stimulation of serum-starved CEF by adding serum (Fig. 1, lower panel). While logarithmically growing or growth-arrested CEF expressed c-Jun as a strong band of 40 kDa, we detected drastic mobility shifts of c-Jun protein after UV irradiation or growth stimulation (Fig. 1). Just after UV irradiation, c-Jun formed a major band of 44 kDa together with three faint bands with apparent molecular weights of 45 kDa, 42 kDa and 40 kDa (Fig. 1, upper panel, 20 min). The shifted bands of endogenous c-Jun remained detectable for at least 320 min. At around 60 min after irradiation, all of these protein bands were much denser than at 20 min, indicating that the synthetic rate of c-Jun increased markedly after UV irradiation. Growth stimulation was also accompanied with c-Jun mobility shifts, though these were transient, being detectable only 20 min after the stimulation. When AP-1 components were purified from UV-irradiated CEF by means of DNA affinity chromatography, all these forms of c-Jun (45-, 44-, 42-, 40-kDa bands) were detected [18], indicating that all of them retain DNA binding activity (data not shown).

Small mobility shifts of c-Jun have been reported to occur in CEF [27–29] or other fibroblasts after UV irradiation, though not after serum stimulation, but such a drastic mobility shift of c-Jun has not been reported before in any fibroblasts. Therefore, we were interested in the molecular mechanisms involved in the production of these unique c-Jun forms and also in their possible biological function. We first examined the possibility of ubiquitination, because the highest mobility shift corresponded to an apparent increase in molecular weight of 5 kDa, which roughly corresponds to mono-ubiquitination. To prepare large amounts of these unique forms of c-Jun, CEF were infected with a virus vector carrying His-tagged c-Jun (wild type) and UV-irradiated. From these cells, more than 1 μg of exogenous c-Jun proteins was purified by Ni-NTA column chromatography and analyzed by Western blotting using anti-ubiquitin antiserum [30]. While this serum

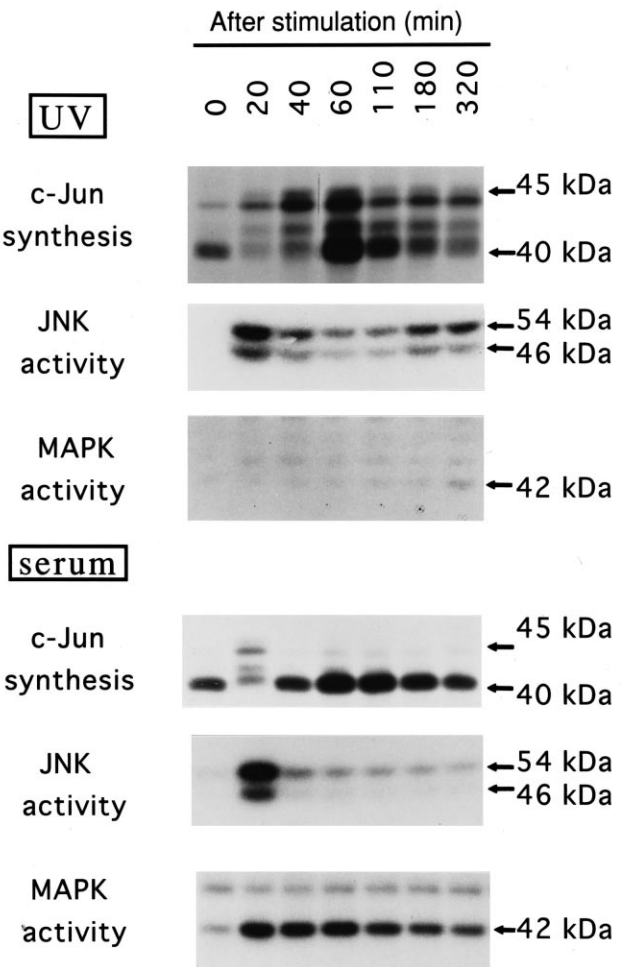

Fig. 1. Patterns of c-Jun biogenesis and endogenous activity of JNK or MAPK after either UV irradiation (UV) or growth stimulation (serum). At time 0, logarithmically growing CEF were UV-irradiated (20 J/cm^2) or growth-arrested CEF were supplemented with CS (final 15%). Whole cell lysates for in-gel kinase assay were prepared at the times indicated. The parallel cultures were pulse-labeled with [^{35}S]methionine for 20 min and cell lysates were prepared under denaturing conditions at the times indicated. Labeled lysates were immunoprecipitated with anti-c-Jun (#14) antiserum, resolved on an SDS-10% polyacrylamide gel and visualized by fluorography (upper). The non-labeled lysates were separated in SDS-10% polyacrylamide gel containing the c-Jun N-terminal region (middle) or MBP (lower). After in-gel kinase reaction, ^{32}P-labeled proteins were detected by autoradiography.

efficiently recognized proteins conjugated with ubiquitin (corresponding to 20 ng of ubiquitin), it failed to detect any c-Jun band at all (data not shown).

Next, to examine the possibility of phosphorylation, the total immunoprecipitate with anti-c-Jun antiserum was prepared from ^{35}S-labeled CEF at 40 min after UV irradiation and treated with bacterial alkaline phosphatase (BAP), potato acid phosphatase (PAP) or calf intestine alkaline phosphatase (CIP). Treatment with BAP or PAP generated only intermediate broad bands (43∼41 kDa) (data not shown), while all of the bands (45, 44, 42 and 40 kDa) were converted to a single band of 40 kDa by CIP treatment (Fig. 2). This conversion by CIP treatment was totally abolished by a competitive inhibitor of alkaline phosphatase, PNPP, indicating that it was due to dephosphorylation, but not proteolysis by a contaminating protease. These results indicate that the c-Jun mobility shifts were exclusively caused by phosphorylation. Since JNKs (JNK1 and JNK2) have been reported to be responsible for c-Jun phosphorylation in response to stress [11–13], we next analyzed the kinetics of the activation of endogenous JNKs as well as ERK2 (the only known MAPK in CEF) [31], which is closely related to JNKs and has been suggested to be a candidate c-Jun kinase. In both UV irradiation and growth stimulation experiments, the density of 44-kDa and 45-kDa bands correlated well with JNK activity and not with MAPK activity, as determined by in-gel kinase assay of parallel cultures (Fig. 1).

3.2. Phosphorylation state of mobility-shifted c-Jun protein forms

To analyze in detail the relationship between the mobility shift and the phosphorylation state, each ^{32}P-labeled c-Jun protein band in PAGE (Fig. 3B, left panel) was excised from the gel and analyzed by comparison of two-dimensional tryptic maps (Fig. 3B, right panel). Because the amino acid sequences of human, mouse, rat and chicken c-Jun proteins are highly conserved, the tryptic peptides containing the JNK phosphorylation sites (consensus sequence; Ser/Thr-Pro) were expected to be assignable on the basis of previous reports on mouse or human c-Jun [32–34]. In the single 40-kDa c-Jun band derived from logarithmically growing CEF labeled with inorganic [^{32}P]phosphate, a clear spot of phosphopeptide 'x' was detected, indicating that one of the representative JNK phosphorylation sites, Ser^{73}, is efficiently phosphorylated even in growing CEF. As in the case of ^{35}S-labeled proteins, four ^{32}P-labeled protein bands with molecular weights of 40 kDa (band 1), 42 kDa (band 2), 44 kDa (band 3) and 45 kDa (band 4) were detected after UV irradiation. Compared with the c-Jun band of non-irradiated CEF (UV−), the density of phosphopeptide 'y' in band 1 was significantly enhanced, approaching that of 'x'. In band 2, phosphopeptide 'v' became detectable, and its relative density was significantly increased in either band 3 or 4, while the relative intensities of spots 'x' and 'y' as well as three spots 'a', 'b' and 'c', corresponding to C-terminal peptides, were little changed among bands 1–4. Phosphoamino acid analysis of bands 1–4 showed that each band was composed of phosphoserine (P-Ser) and phosphothreonine (P-Thr) in various ratios (Fig. 3C). Band 1 contained predominantly P-Ser, while similar amounts of P-Ser and P-Thr were detected in bands 3 and 4, and the P-Ser/P-Thr ratio in band 2 was intermediate between those of bands 1 and 3. Therefore phosphopeptides in spot 'v' were expected to be mainly composed of P-Thr, consistent with the previous report that the peptides that migrated to the similar position to spot 'v' were phosphorylated on Thr^{91} and/or Thr^{93} [11,12,34]. For the drastic mobility shifts to 44 kDa and 45 kDa, therefore, phosphorylation of both Thr^{91} and Thr^{93} seemed to be essential in addition to the phosphorylation of Ser^{63} and Ser^{73}.

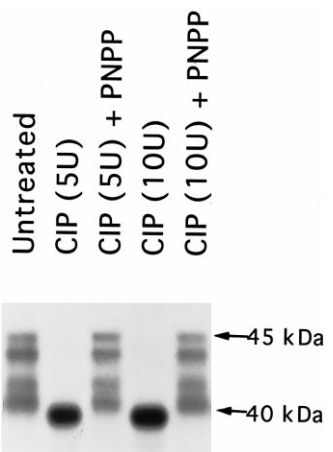

Fig. 2. Dephosphorylation of c-Jun proteins isolated from UV-irradiated CEF by calf intestinal alkaline phosphatase (CIP) treatment. Logarithmically growing CEF were UV-irradiated and 20 min later they were pulse-labeled with [^{35}S]methionine for 20 min. Whole cell lysates were prepared under denaturing conditions and immunoprecipitated with anti-c-Jun (#14) antiserum. The immunoprecipitates were solubilized and split into five aliquots. They were incubated for 1 h at 37°C in the absence (lane 1) or presence of 5 U (lanes 2 and 3) or 10 U (lanes 4 and 5) of CIP with (lanes 3 and 5) or without (lanes 2 and 4) phosphatase inhibitor (10 mM *p*-nitrophenyl phosphate; PNPP).

3.3. Analysis of substitution mutants at Ser^{63} and Ser^{73}

In an attempt to increase the levels of the mobility-shifted forms of c-Jun, we constructed a virus vector encoding JNK1 and introduced it into CEF. However, although JNK1 expression was induced, no elevation of JNK activity was detected in these non-transformed cells (data not shown), indicating that qualitative change rather than quantitative change is essential for elevation of JNK activity. Therefore, we next prepared substitution mutants at Ser^{63} and Ser^{73} to analyze the biochemical and biological functions of the phosphorylation of these two serine residues (Fig. 4A). In one mutant, SSAA, both of these serine residues were substituted with alanine to prevent phosphorylation, while in the other mutant, SSDD, the same residues were replaced with aspartic acid to mimic phosphoserine. These two mutants as well as wild-type c-Jun, were fused with a histidine tag at the N-terminus and introduced into CEF using replication-competent retrovirus vectors. To compare the mobility shifts of these mutants with those of the wild type, total cell extracts were prepared from logarithmically growing CEF infected with these virus vectors before or after UV irradiation. Before UV irradiation, c-Jun wild type and its two mutants migrated as a single band. While drastic mobility shifts were observed in the wild type after UV irradiation, such mobility shifts were completely abolished in SSAA. SSDD in growing CEF had an apparently slightly higher molecular weight than wild-type c-Jun, but UV irradiation did not cause the full mobility shifts. From these

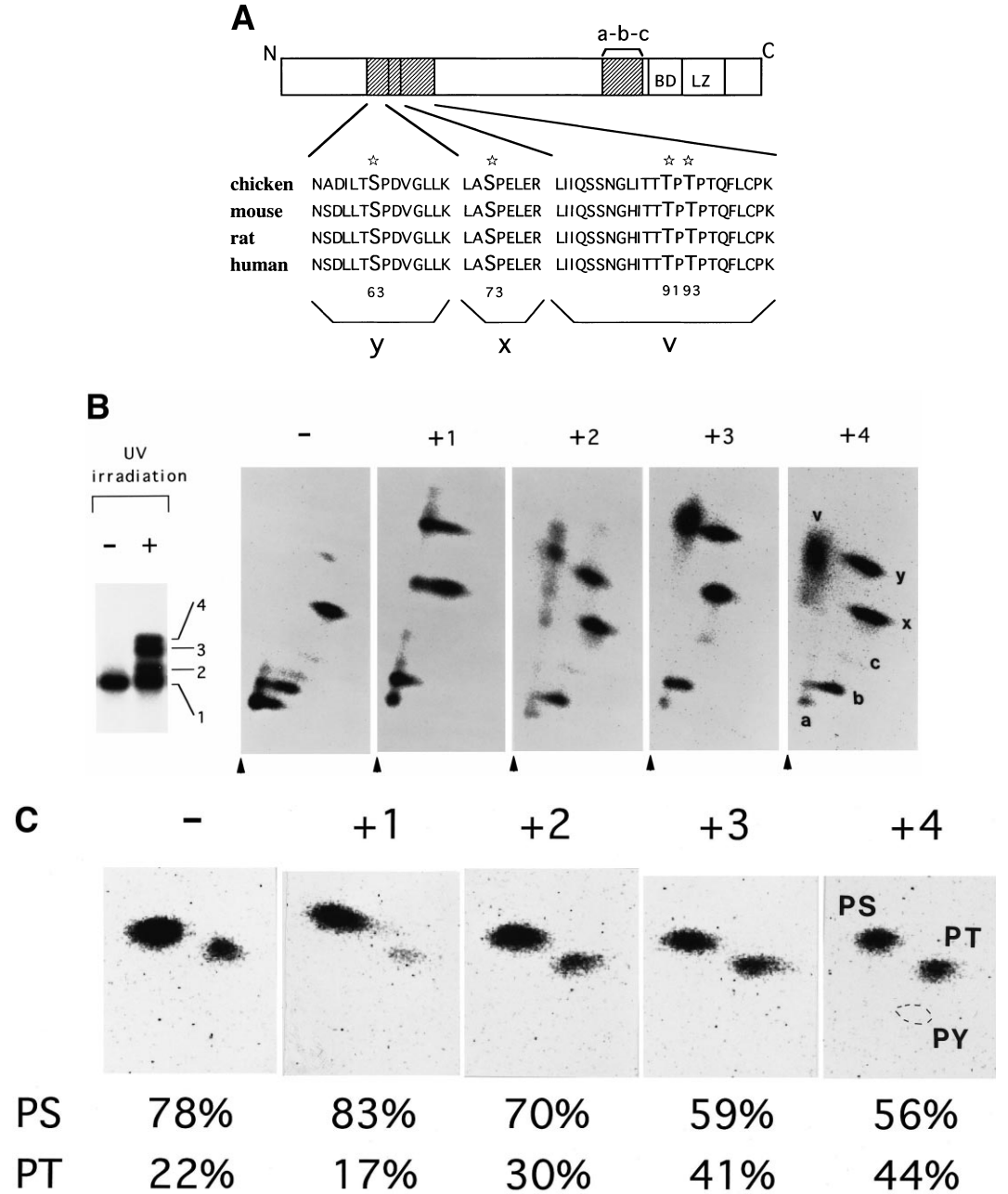

Fig. 3. Phosphorylation patterns of c-Jun proteins detected in UV-irradiated CEF. A: Schematic representation of c-Jun protein. Numbers refer to amino acid residues. Individual tryptic peptides ('y', 'x', 'v', and 'a-b-c') and phosphorylatable residues (bold letters) are indicated. Amino acid sequences of the tryptic peptides are compared among chicken, mouse, rat, and human c-Jun. BD: basic domain; LZ: leucine zipper motif. Shaded boxes indicate phosphorylatable tryptic peptides. B: Two cultures of logarithmically growing CEF were pulse-labeled for 120 min and one (+) was UV-irradiated at 80 min after the start of labeling while the other (−) was untreated. Whole cell lysates were immunoprecipitated with anti-c-Jun (#14) antiserum and the immunoprecipitates were resolved on SDS-10% polyacrylamide gel and detected by autoradiography (left panel). Each of the c-Jun protein bands shown here was excised and analyzed by two-dimensional tryptic phosphopeptide mapping. The ^{32}P-labeled peptides were visualized by Bio-Imaging Analyzer (right panel). C: A part of each c-Jun protein band shown in B, left panel, was subjected to partial acid hydrolysis and analyzed by two-dimensional electrophoresis as described previously [26]. The phosphoamino acids were visualized and their relative radioactivity was measured by Bio-Imaging Analyzer. In the lower panel, the percentage of phosphoserine and phosphothreonine is shown. PS: phosphoserine; PT: phosphothreonine; PY: phosphotyrosine.

results, phosphorylation of Ser^{63} and Ser^{73} is required for the drastic mobility shifts although it is not sufficient for them, as we concluded in the previous section.

As shown in Fig. 5, CEF fully infected with c-Jun (rat) virus assumed an elongated shape compared with those infected with the control vector. The virus stocks formed foci at the titer of more than 6×10^5 focus forming units/ml and formed colonies in soft agar at a similar efficiency as that for c-Jun (human) virus [6], clearly indicating its transforming activity. CEF infected with SSAA virus showed very similar cellular morphology to that of CEF expressing c-Jun wild type (Fig. 5) and retained similar efficiency of colony forma-

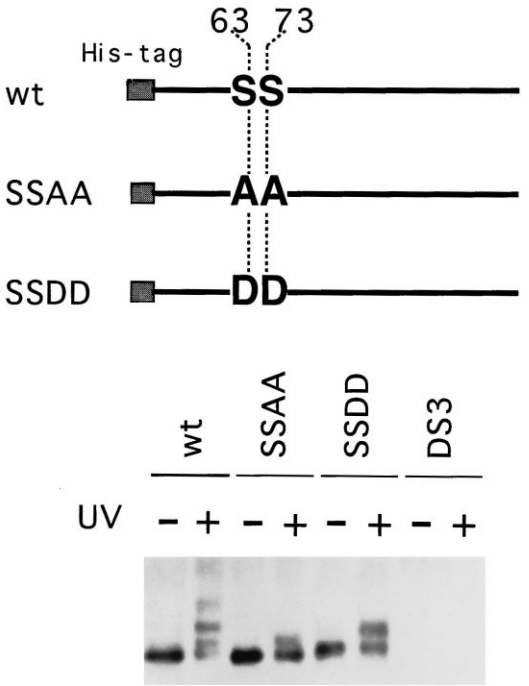

Fig. 4. A: Schematic representation of c-Jun wild type (wt) tagged with His at the N-terminal or its mutants substituted at both serines 63 and 73 with alanine (SSAA) or with aspartic acid (SSDD). B: His-tagged c-Jun wt and its substitution mutants were introduced into CEF using retrovirus vectors. CEF infected with the control vector (DS3) were also prepared. One of each pair of cultures (+) was UV-irradiated 40 min before the preparation of cell lysates, while the other (−) was untreated. Whole cell lysates were analyzed by Western blotting using affinity-purified anti-c-Jun (#10) antiserum. Under these conditions, endogenous c-Jun protein was not detected.

tion in soft agar, in accordance with a previous report using a mouse c-Jun mutant, c-Jun^{ALA} [35]. These results indicate that the unique forms of c-Jun with high mobility are not essential for the cellular transformation. It was further confirmed by our observation that c-Jun (human) that was exogenously expressed in CEF by the retrovirus vector did not contain spot 'v' after phosphotryptic mapping (data not shown) like endogenous c-Jun (UV−, Fig. 3B, left panel). Furthermore, the transforming phenotype of SSDD virus-infected CEF was not significantly enhanced compared with that of c-Jun (wt) virus-infected CEF as judged from the cellular morphology and anchorage-independent growth.

In this report, we have shown that the unique forms of c-Jun with apparent molecular weights of 45 kDa and 44 kDa detected in CEF represent forms extensively phosphorylated by activated endogenous JNKs. This unique feature of c-Jun in CEF may partly reflect the relatively high level of activity of JNKs, which phosphorylate c-Jun at Ser^{73} even in normally growing CEF. CEF always have higher endogenous JNK activity than NIH3T3, or human tumor cell lines so far examined, whether logarithmically growing cells or UV-stimulated cells are compared (our unpublished results), and this provides further support for our ideas. Our mutational analysis, however, did not provide any evidence that the highly phosphorylated forms of c-Jun contribute to cellular transformation.

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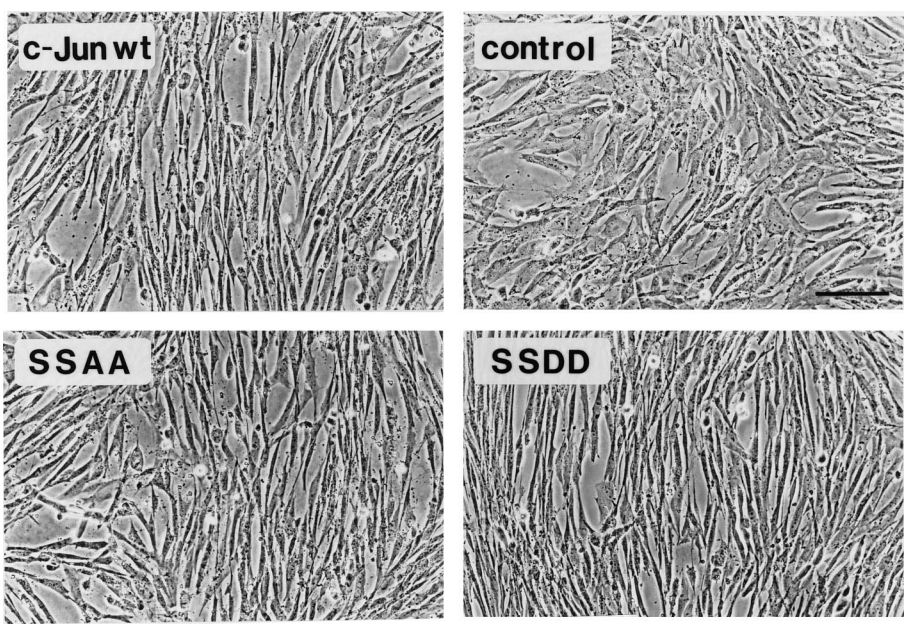

Fig. 5. Cellular morphology of CEF expressing c-Jun wild type or c-Jun substitution mutants. CEF were infected with DS3 (control vector), or with vector encoding wtJ (c-Jun wt), SSAA, or SSDD and kept under soft agar for four days before the photographs were taken under a phase-contrast microscope. The bar corresponds to 100 μm.

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