Biol. Chem., Vol. 380, pp. 569–578, May 1999 · Copyright © by Walter de Gruyter · Berlin · New York

Enzyme-Linked Immunosorbent Assay for the Measurement of JNK Activity in Cell Extracts

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A colorimetric enzyme-linked immunosorbent assay (ELISA) for the measurement of kinase activity of c-Jun N-terminal kinases (JNKs) in cell extracts is described. The assay involves passive immobilisation of the substrate GST-cJun on the surface of a microtiter plate, selection of JNK protein kinases directly in substrate-coated wells, kinase reaction, and detection of substrate phosphorylation by a phosphoepitope-specific antibody. The ability of this assay to selectively measure JNK activity relies on the high-affinity interaction between JNKs and c-Jun. Accordingly, we found that JNKs could be captured on the microtiter plate surface through binding to the immobilised GST-cJun. Moreover, JNKs retained the specificity of their interaction with and phosphorylation of c-Jun with respect to the dependence on both intact docking domain and the dimerisation state of c-Jun. This novel procedure represents a marked improvement on conventional radioactive assays in terms of sensitivity, accuracy of evaluation, low time consumption, high throughput and amenability to automation. It is expected to be useful for the acceleration and facilitation of JNK activity measurement in cell extracts, in particular for large-scale screening of clinical samples.

Key words: ELISA / Kinase assay / MAPK / Protein immobilisation.

Introduction

The c-Jun N-terminal kinases (JNKs), also referred to as stress-activated protein kinases (SAPKs), are proline-directed serine/threonine kinases that represent one of the five currently known subclasses of the mitogen-activated protein kinase (MAPK) superfamily. The JNK group comprises ten isoforms arising from alternative splicing of three distinct JNK gene transcripts (Gupta *et al.*, 1996).

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Activation of JNKs is achieved by dual phosphorylation at Thr and Tyr within a TPY motif which is distinct from corresponding motifs in other MAP kinases. The JNK signal transduction pathway is stimulated in response to a wide array of cellular stresses, as well as by the engagement of certain classes of cell surface receptors, including several cytokine receptors, receptor tyrosine kinases, and serpentine receptors (reviewed in Whitmarsh and Davis, 1996). The importance of JNK protein kinases is underlined by their implication in a plethora of physiological processes such as hematopoiesis (Hu *et al.*, 1996; Kiefer *et al.*, 1996; Nishina *et al.*, 1997), oncogenic transformation (Raitano *et al.*, 1995; Cavigelli *et al.*, 1996; Xu *et al.*, 1996; Frey and Mulder, 1997), ischemia (Pombo *et al.*, 1994), T cell activation (Su *et al.*, 1994), leukocyte emigration (Read *et al.*, 1997), apoptotic cell death (Xia *et al.*, 1995; Verheij *et al.*, 1996; Chen *et al.*, 1996; Zanke *et al.*, 1996a; Ichijo *et al.*, 1997; Yang *et al.*, 1997; Goillot *et al.*, 1997), and both early embryonic development and immune response in *Drosophila* (Sluss *et al.*, 1996; Riesgo Escovar *et al.*, 1996). Many investigators aim to characterise the role of JNKs in such processes or to uncover new modes of action of this pathway. Thus, a rapid, sensitive, high-throughput assay for measurement of JNK activity in cell extracts would be of great value.

Conventional radioactive JNK assays comprise three basic protocols which ensure the specificity of JNK measurement:

(i) a frequently used immunocomplex assay employing isoform-specific antibodies (Yan *et al.*, 1994);
(ii) a solid-phase assay based on specific interaction between JNKs and c-Jun (Hibi *et al.*, 1993); and
(iii) an in-gel assay relying upon recovering of JNK activity by renaturation after SDS-gel electrophoresis (Hibi *et al.*, 1993).

These JNK assays use the native protein substrate c-Jun, often fused to glutathione S-transferase (GST), which is phosphorylated during the kinase reaction employing [^{32}P]ATP. After substrate (i and ii), or kinase (iii) separation by gel electrophoresis, substrate phosphorylation is assessed by autoradiography. These procedures are undoubtedly sufficiently specific, but they also possess several drawbacks particularly noticeable in high-throughput screening: they are laborious, not amenable to automation and require ^{32}P, a high-energy β-emitter which needs special handling, generates radioactive waste, and has a half life of only 14 days. This considerably restricts the flexibility of these procedures. Moreover, these assays are carried out at subphysiological levels of ATP owing to the necessity of keeping [^{32}P]ATP levels within experimentally permissive limits.

To bypass the limitations of traditional radioactive procedures, several ELISA-based assays for the measurement of activity of recombinant and purified protein tyrosine kinases have been developed (Cleaveland *et al.*, 1990; Lazaro *et al.*, 1991; Farley *et al.*, 1992; Angeles *et al.*, 1996; Cheng and Hui, 1996; Lehel *et al.*, 1997). In general, these procedures use immobilised synthetic peptide or polymer substrates which are phosphorylated by the kinase of interest. The phosphorylation of substrate is evaluated by detection with a phosphotyrosine antibody and an appropriate secondary antibody conjugate. Unfortunately, the peptide substrates cannot be used in MAP kinase assays because of the stringent requirements of these enzymes for native protein substrates (Robinson *et al.*, 1996). Therefore, we recently developed an ELISA-based kinase assay for ERK2, JNK2, and p38 MAP kinases that employs the immobilised GST-conjugated protein substrates Elk-1, c-Jun, and ATF2, respectively (Forrer *et al.*, 1998). This rapid nonradioactive procedure makes use of phosphoepitope-specific antibodies that selectively recognise the sites phosphorylated by individual MAP kinases. We found that recombinant MAP kinases mostly retain their specificity in phosphorylation of immobilised substrates, although hydrophobic adsorption of such immobilised proteins might result in severe alterations in their structure (Schwab and Bosshard, 1992; Butler *et al.*, 1992; Houen and Koch, 1997). Since the phosphorylation of immobilised substrates fulfilled also all structural requirements for such a reaction, we concluded that MAP kinases retained their ability to undergo specific bipartite interactions with the respective substrates.

The binding of JNKs to their substrate c-Jun has recently been found to be a prerequisite for efficient phosphorylation (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kallunki *et al.*, 1994). This binding was ascribed to a short docking region of c-Jun (termed δ domain) adjacent to the phosphoacceptor sites. Both active and inactive JNKs associate with c-Jun; however, phosphorylation of c-Jun by active JNKs results in dissociation of the c-Jun–JNK complex (Hibi *et al.*, 1993; Dai *et al.*, 1995). The ability of JNKs to bind c-Jun was traced to a small β-strand-like region near the catalytic pocket of these enzymes (Kallunki *et al.*, 1994). This specificity-determining region located between subdomains IX and X also represents a region with the major sequence differences between individual JNK isoforms, thereby explaining their different binding affinities to c-Jun (Gupta *et al.*, 1996). Similar docking interactions between JNKs and a distinct binding domain appear to direct the phosphorylation of transcription factors ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Gupta *et al.*, 1996), ATFa (Bocco *et al.*, 1996), Elk-1 (Gille *et al.*, 1995), and NFAT4 (Chow *et al.*, 1997).

By employing such specific, high-affinity interactions as a selection tool for JNKs, we aimed in this work at developing an ELISA-based assay for measurement of JNK activity in cell extracts. We found that JNK protein kinases could be captured on the microtiter plate surface through their binding to the immobilised GST-Jun. The interactions between JNKs and the immobilised substrate retained their specificity with respect to the dependence on both intact docking domain and the dimerisation state of the substrate, and therefore conferred specificity to the kinase selection step of this assay. Finally, the usage of a phosphoepitope-specific c-Jun antibody, that selectively recognises the sites phosphorylated by JNKs, provided an additional level of specificity to the measurement of JNK activity by this novel method.

Results

We recently developed an ELISA-based procedure for the nonradioactive measurement of kinase activity of recombinant JNK kinases (Forrer *et al.*, 1998). In order to find a convenient surrogate method for the tedious measurement of JNK activity in cell extracts by immunocomplex assay, we further sought to merge the convenience of ELISA technology with tools for selection of JNKs. We describe here a novel colorimetric ELISA-based JNK assay which employs immobilised GST-cJun (aa 1-79), a well-characterised substrate of JNKs, and takes advantage of the highly specific interaction between JNKs and c-Jun for the selective enrichment of cellular JNKs on the surface of a microtiter plate. The substrate phosphorylation in this assay is detected by a phosphoepitope-specific antibody recognising c-Jun phosphorylated on Ser73, and, upon incubation with the secondary antibody conjugated to alkaline phosphatase, the colour development is monitored by measuring the absorbance at 410 nm. The experimental conditions of all steps of this assay have been optimised and are incorporated in the standard protocol described in Materials and Methods. Although all experiments were done with lysis buffer containing phosphate

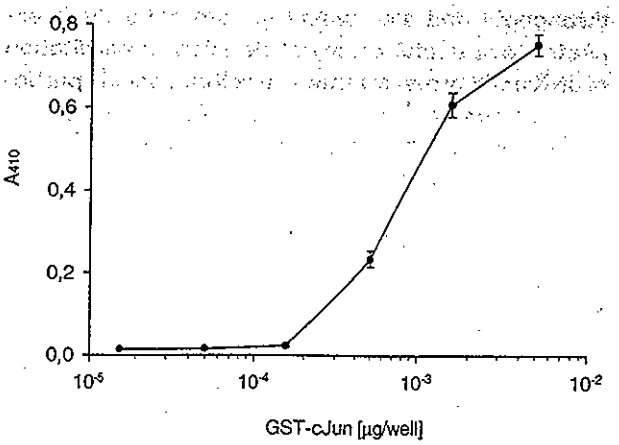

Fig. 1 Dependence of Signal on the Substrate Coating Concentration.
Wells were coated for 2 h at 37 °C with the indicated concentration of GST-cJun diluted in TBS. 50 µg/well of extract from starved NIH3T3 irradiated with 200 J/m^2 UV-C were added and incubated overnight at 4 °C. ELISA was performed as described in Materials and Methods. Each data point represents the mean of three parallel determinations ± s.e.m. from one ELISA experiment.

buffered saline, Tris buffered saline can be used equally well. This assay tolerated also higher concentrations of the nonionic detergents Nonidet P-40 (1%; w/v), Triton X-100 (0.1%; w/v), and Tween-20 (0.1%; w/v). However, commonly used components of lysis buffers like the ionic detergent SDS (0.1%; w/v) or the reducing agent DTT (0.5 mM) led to a drastic reduction of the obtained signals. Additives like Mg^{2+}, which might facilitate an illegitimate phosphorylation of the substrate during the incubation with cell extract, should also be omitted from the lysis buffer (data not shown).

In order to determine the optimal substrate concentration for plate coating, the signal was measured in response to increasing GST-cJun concentrations. As shown in Figure 1, the signal intensity continuously increased within the concentration range used in this experiment. To keep the substrate consumption low, the GST-cJun concentration of 0.5 μg/well was chosen for all subsequent experiments.

To provide evidence that no illegitimate phosphorylation of GST-cJun took place during the overnight incubation with the cell extract at 4 °C, we examined the time course and the ATP dependence of the kinase reaction. We found that the reaction rate of GST-cJun phosphorylation was constant for 120 min, and that the kinetics curve intersected the ordinate at the zero point (Figure 2A). This result suggested that the substrate phosphorylation was confined to the kinase reaction step of this assay. As shown in Figure 2B, the kinase activity increased also in an ATP-dependent manner, and the response was saturable. The apparent K_m value for selected kinases (14 ± 2 μM) corresponded well with our previous results obtained with recombinant JNK2 (Forrer *et al.*, 1998). Moreover, convergence of the ATP dose response curve with the abscissa, i.e. the absolute requirement for extraneous ATP, again ruled out the possibility of any illegitimate phosphorylation of GST-cJun prior to the kinase reaction step. It is worth pointing out in this context that the assays using radioactive tracer ATP are inefficient at ATP concentrations close to the physiological millimolar levels, unless a large amount of radioactive ATP is added. In contrast, for this ELISA-type of assay, no substantial loss of sensitivity was observed in the millimolar range.

Next, we compared the signal-to-enzyme concentration relationship for the JNK activities present in extracts from NIH3T3 cells that had been irradiated with UV-C or left untreated. Both kinase reactions were linear with respect to the protein content over at least one order of magnitude (Figure 3A). As expected, an approximately ten-fold increase in the extent of GST-cJun phosphorylation was observed in extracts from UV-treated cells. As determined by immunoblotting, the levels of JNK1 and JNK2 proteins remained unchanged after UV-C treatment (data not shown). We also tested whether recombinant, fully-active JNKs (Khokhlatchev *et al.*, 1997) were able to deliver signals comparable with their cellular counterparts. Indeed, signals generated by as little as 5 ng of recombinant JNKs could be detected (data not shown).

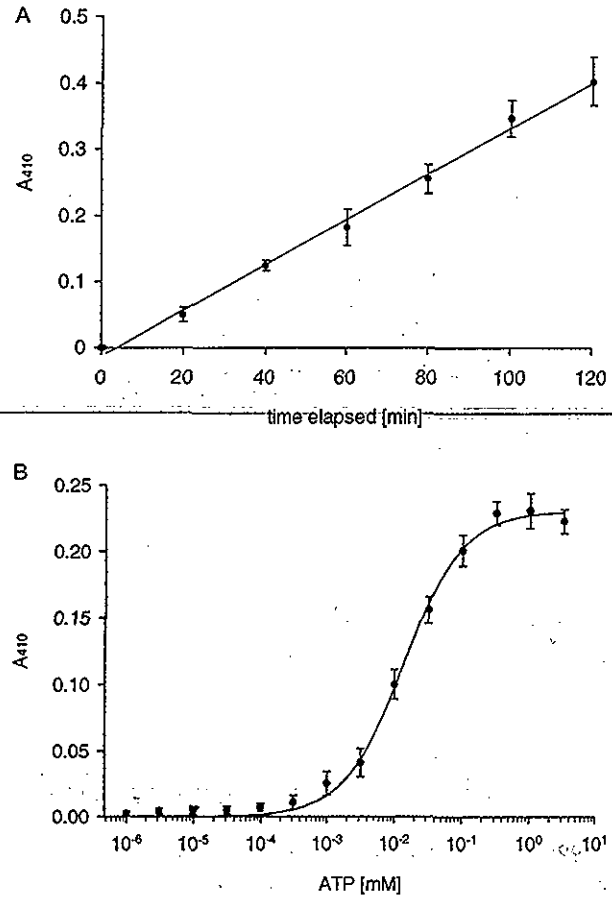

Fig. 2 Time Course and ATP Dependence of the Phosphorylation Reaction.
(A) Kinase buffer without ATP was used at the beginning of the 120 min incubation period. Then, at 20 min intervals, ATP was added to a final concentration of 1 mM, and the kinase reaction was allowed to proceed for indicated time periods. (B) The effect of ATP on the phosphorylation of GST-cJun was examined by varying the ATP concentration in the kinase buffer. The data were fitted by nonlinear regression into the Michaelis-Menten equation using SigmaPlot (Jandel, San Rafael, CA, USA), and the apparent K_m value of 14 ± 2 μM was obtained. In both experiments 50 μg/well of extract from UV-treated NIH3T3 cells were added and incubated overnight at 4 °C, and ELISA was performed as described in Materials and Methods. Each data point in (A) and (B) is the mean *of three parallel determinations ± s.e.m. from one ELISA experiment.*

Although not necessary for most applications, we next sought to reduce the amount of extract and the length of incubation required for the binding of sufficient quantities of JNK molecules to generate detectable signals. To pursue this task, we took advantage of favourable properties of polyethylene glycol (PEG), a water-soluble, hydrophobic and nondenaturing polymer, which is known to cause macromolecular crowding, and consequently to facilitate the interaction of binding partners (Maehara *et al.*, 1985; Harrison and Zimmerman, 1986). As shown in Figure 3B, by using 15% PEG 8000 we were able to reduce both the amount of extract and the incubation time necessary for efficient JNK binding about five-fold. Nevertheless, because of problems with the protein precipitation encoun-

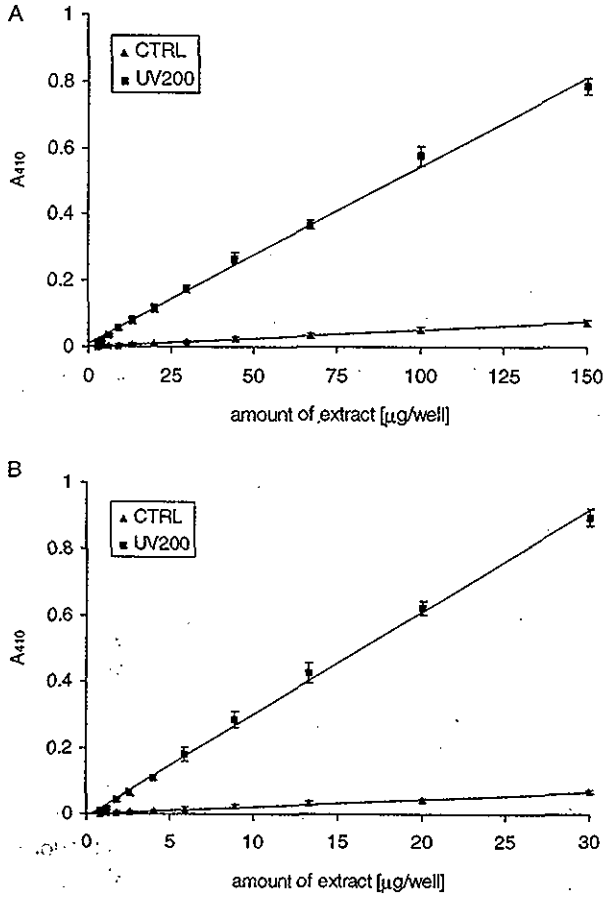

Fig. 3 Linearity of the JNK Assay.
(A) Extracts from untreated or UV-irradiated (200 J/m^2) starved NIH3T3 cells were diluted several times by a factor 1.5 and incubated overnight at 4 °C. (B) Extracts from untreated or UV-irradiated (200 J/m^2) starved NIH3T3 cells were supplemented with an equal volume of lysis buffer containing 30% PEG 8000, diluted several times by factor 1.5 and incubated overnight at 4 °C. ELISA was performed as described in Materials and Methods. Each data point in (A) and (B) is the mean of three parallel determinations ± s.e.m. from one ELISA experiment.

tered at higher protein concentrations, PEG is to be used with caution.

In order to examine the specificity of this JNK assay, we attempted to deplete individual JNK isoforms from the cell extracts. A polyclonal antibody recognising JNK1 (C-17, Santa Cruz), that was able to deplete this isoform completely (as judged by immunoblotting), caused up to 50% reduction of the measured kinase activity (data not shown). Unfortunately, no JNK2-specific antibody available to us could deplete the extracts of this isoform which presumably accounts for the residual phosphorylation of GST-cJun.

To further analyse the specificity of this assay, we examined whether the kinase binding during the selection step retained its dependence on the docking domain of c-Jun which is distinct from, and located N-terminally to, the phosphoacceptor sites. For this purpose, we compared the phosphorylation rate of GST-cJun (aa 1–79) with the phosphorylation rate of GST-ΔcJun (aa 43–223) which lacks the critical N-terminal residues. As shown in Figure 4A, the selected kinases exhibited a strong preference for the substrate with the intact docking domain. We previously demonstrated that the deletion of this domain leads to an approximately four-fold reduction in the rate of phosphorylation of immobilised GST-cJun by recombinant JNK2 (Forrer *et al.*, 1998). Here, the effect of this deletion was substantially larger. Thus, besides its role in phosphorylation, the docking domain of c-Jun appears to be important also for the capturing of JNKs during the selection step of this assay. Since both natural JNK substrates and the GST fusion proteins are dimers (Lim *et al.*, 1994), we were interested whether the dimerisation contributes to the binding and phosphorylation of c-Jun by JNKs. We therefore fused c-Jun and Δc-Jun with the monomeric bacteriophage lambda protein D (pD) (Forrer and Jaussi, 1998). As shown in Figure 4A, these monomeric substrates appeared to be entirely ineffective in the binding

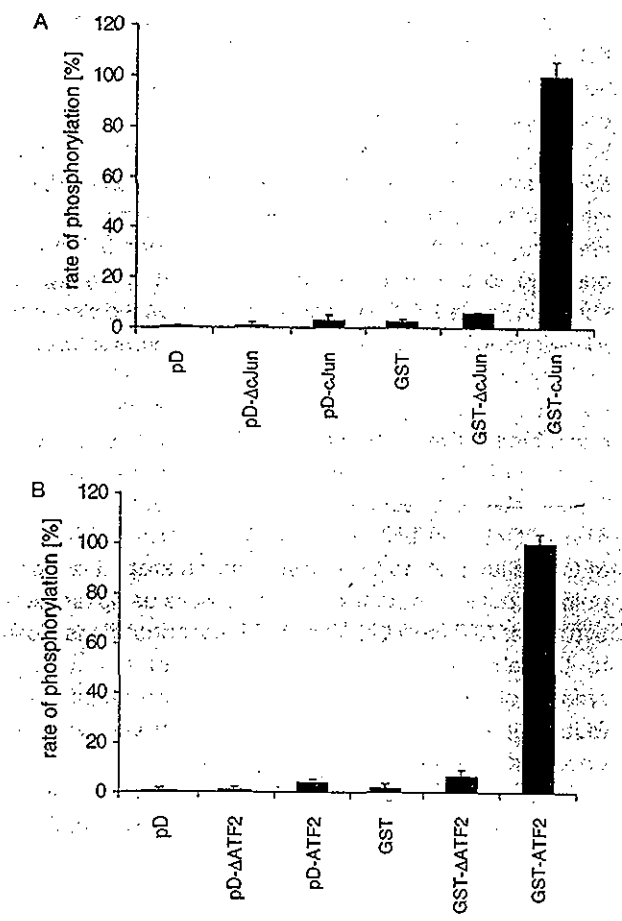

Fig. 4 Influence of N-terminal Docking Domain and of Fusion Partner on the Binding and Phosphorylation of JNK Substrates.
The wells were coated with 0.5 µg/well of GST-cJun (A) or GST-ATF2 (B), or with equimolar amounts of other fusion proteins as described in the text. Subsequently, 50 µg/well of extract from UV-treated NIH3T3 cells were added and incubated overnight at 4 °C, and ELISA was performed as described in Materials and Methods. The rate of phosphorylation obtained for GST-cJun or GST-ATF2 was set to 100% for each data set. Each data bar in (A) and (B) is the mean of three parallel determinations + s.e.m. from one ELISA experiment.

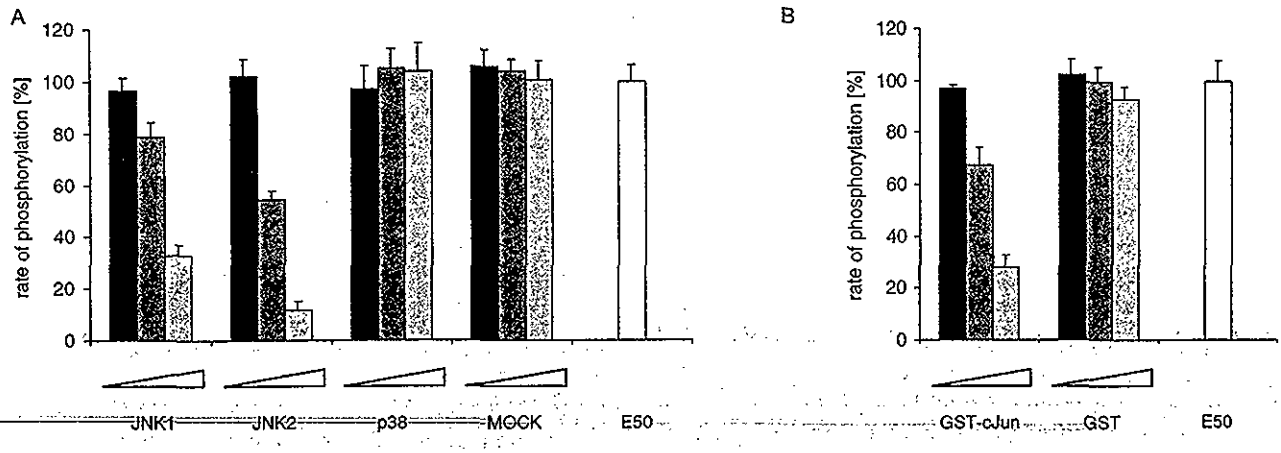

Fig. 5 Specificity of the Kinase-Substrate Interaction.
(A) The competition assay with inactive recombinant JNK1, JNK2, and p38 kinases was performed as described in Material and Methods. Briefly, the kinases were dephosphorylated by lambda phosphatase, and thereafter 1, 10, and 50 μg/well of JNK1, or equimolar amounts of JNK2, p38, or only lambda phosphatase (mock) were allowed to pre-bind in the GST-cJun coated microtiter plate for 12 h at 4 °C. Subsequently, 50 μg/well of extract from UV-treated NIH3T3 cells (E50) were added and incubated for additional 12 h at 4 °C. The rate of phosphorylation obtained for E50 was set to 100%. (B) The extracts from UV-treated NIH3T3 cells were preincubated with 2, 20 and 200 μg/ml of recombinant GST or GST-cJun for 2 h on ice. Afterwards, the mixtures were incubated on GST-cJun coated microtiter plate overnight at 4 °C, and ELISA was performed as described in Materials and Methods. Each data bar in (A) and (B) is the mean of three parallel determinations + s.e.m. from one ELISA experiment.

and/or capability of being phosphorylated by cellular JNKs, and again, this effect surpassed many-fold our previous observations obtained with recombinant JNK2. Finally, since the transcription factor ATF2 is phosphorylated by JNKs with an efficiency comparable to c-Jun, we also examined the structural requirements for the immobilised ATF2-derived substrates. As shown in Figure 4B, analogously to c-Jun, we observed the same dependence on both intact N-terminal docking domain and the dimerisation state of this substrate. Collectively, these data point to the necessity of substrate dimerisation *per se*, as a prerequisite for binding and phosphorylation of substrates by JNKs.

Due to diffusion-rate control and a slow off-rate, reactions between binding partners on solid phase seldom reach equilibrium within the time range used for ELISA experiments (Nygren and Stenberg, 1985; Franz and Stegemann, 1991; Nygren and Stenberg, 1991). Therefore, the rules for competitive binding of reagents in solution phase, where the reaction is reversible and not limited by diffusion, do not apply to the nonequilibrium solid phase conditions. Consequently, it is not surprising that the amount of reagents required for efficient competition on solid phase is usually considerably higher than the amount required under equivalent solution phase conditions. Despite this fact, we decided to further analyse the assay specificity by conducting competition experiments with inactivated recombinant kinases. We singled out two representative JNK isoforms–JNK1α1 and JNK2α2, which have been reported to display a significant difference in binding to c-Jun (Gupta *et al.*, 1996), and a related protein kinase p38α, which is known to be activated by almost identical stimuli as the JNK pathway, and might, therefore, be detrimental for the specific measurement of JNK activity in this assay. As shown in Figure 5A, with increasing concentrations both JNK1α1 and JNK2α2 competed efficiently with cellular JNKs for GST-cJun binding, although, as expected, JNK2α2 was the better competitor. In contrast, p38α kinase was even at high concentrations entirely unable to compete for GST-cJun. These results imply that the activity of some JNK isoforms is, due to tighter binding to c-Jun, detected preferentially over the activity of others, and that p38 kinase does not interfere with the measurement of JNK activity in this assay.

Finally, we tested whether the kinase selection is specific for c-Jun. As shown in Figure 5B, in contrast to GST alone, the added recombinant GST-cJun efficiently competed with the immobilised substrate for the binding to JNKs from cell extract. Hence, the kinase selection in this assay is indeed mediated by the binding to c-Jun and is not attributable to any unspecific interaction.

Discussion

Originally, JNK protein kinases were identified as a novel subgroup of the MAP kinase superfamily that bind to the c-Jun transactivation domain, phosphorylate it on Ser63 and Ser73, and are potently activated in response to the oncoprotein Ha-Ras and UV irradiation (Hibi *et al.*, 1993; Derijard *et al.*, 1994). Over the past several years, the overwhelming complexity of activating stimuli and the extensive ramification of upstream signalling elements became apparent. Many investigators aim at characterising the role of JNKs in various physiological processes or to uncover new modes of action of this pathway. Thus, a rapid, sensitive, high-throughput assay for measurement of JNK activity in cell extracts would be of great value.

Traditional radioactive assays, although sufficiently specific, possess several drawbacks which make them unattractive especially for large-scale screening. In particular, low throughput, laboriousness, hazards associated with waste disposal and handling of radioisotopes, as well as the high amount of cell extract required for a single analysis, i.e. low sensitivity, are the major obstacles hindering widespread use of these assays. In addition, radioactive procedures use subphysiological ATP and supraphysiological substrate concentrations, and are often accompanied by evaluation problems such as high background, and nonlinearities introduced by autoradiography and photographic processing.

In contrast, the ELISA-type of JNK assay described here avoids all of the above difficulties. It is straightforward, precise, well suited for high-throughput screening and is not encumbered by inaccuracy in evaluation. We demonstrated that the phosphorylation of the substrate is confined to the kinase reaction step, and that no illegitimate phosphorylation by unrelated kinases took place during the kinase selection step, i.e. during the incubation with cell extract. Although for sensitivity reasons we were not able to directly monitor the binding of cellular JNKs to GST-cJun, we demonstrated by competition experiments that the kinase selection is mediated by the specific binding of JNKs, particularly JNK2 isoform, to c-Jun. At the same time, this binding proved to be dependent on the intact docking domain of c-Jun which is known to deliver a major contribution to binding of JNKs to c-Jun. Furthermore, the linearity of this assay with respect to the amount of extract and the time course of the kinase reaction ensures an accurate evaluation. Finally, the sensitivity of this procedure surmounts those of radioactive assays up to 100-fold; whereas about 1 mg of total protein is usually required in the radioactive procedures, here as little as 10 μg sufficed to generate a satisfactory signal (an amount that permits repeated analysis of biopsy samples). Moreover, the sensitivity of the ELISA procedure might be further improved by employing chemiluminescent instead of colorimetric detection.

As stated above, the specificity in this JNK assay is conferred by the high-affinity interaction between JNKs and their substrate c-Jun. Such interactions are a hallmark of MAP kinase signal transduction cascades. Recently, several consecutive members of these cascades were reported to associate with each other. The reciprocal interactions between MAPKK kinases and activating GTPases (Teramoto *et al.*, 1996; Fanger *et al.*, 1997), MAPK kinases and MAP kinases (Sanchez *et al.*, 1994; Zanke *et al.*, 1996b; Fukuda *et al.*, 1997), as well as various members of the MAPK family with a number of adaptor, scaffold, or even inhibitor proteins (Choi *et al.*, 1994; Marcus *et al.*, 1994; Kharbanda *et al.*, 1995; Marti *et al.*, 1997; Posas and Saito, 1997; Whitmarsh *et al.*, 1998) have been proposed to assure the specificity within these signal transduction pathways. For kinase assays, however, the specific interactions between substrates and the respective MAPKs may be particularly valuable. In addition to the interaction with c-Jun, JNKs are known to associate with several other transcription factors, e.g. ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Gupta *et al.*, 1996), ATFa (Bocco *et al.*, 1996), Elk-1 (Gille *et al.*, 1995), NFAT4 (Chow *et al.*, 1997), and c-Rel (Zanke *et al.*, 1996b), and to date, a distinct domain responsible for the binding to JNKs has been described for ATF2, Elk-1, and NFAT4. Similarly, ERK MAP kinases have also been reported to associate with their substrates c-Myc (Gupta and Davis, 1994), Elk-1 (Yang *et al.*, 1998b), Spi-B (Mao *et al.*, 1996), p90rsk (Hsiao *et al.*, 1994), and Mnk1 and Mnk2 (Waskiewicz *et al.*, 1997). Moreover, the targeting of ERK MAP kinases to their substrates Elk-1 and c-Myc appears to be directed by specific interactions with distinct docking domains, although in the case of Elk-1, the modes of binding to ERK and JNK are different (Yang *et al.*, 1998a). Therefore, analogously to c-Jun in the procedure presented here, Elk-1-based recombinant proteins might be particularly good candidates for serving as selective substrates in an assay monitoring ERK activity. In general, the employment of specific kinase-substrate interactions in ELISA-based kinase assays would be superior to selection by specific antibodies as used in radioactive immunocomplex assays. Apart from the low sensitivity and laboriousness of such procedures, the use of antibodies for kinase selection might lead to a high background owing to cross-reactivity in ELISA.

The applicability of ELISA-based kinase assays is limited by their semiquantitative nature with respect to the determination of the amount of phosphate incorporated into substrate. Accordingly, the assay presented here can be used for comparison of JNK activities in different samples; however, the day-to-day variations, a factor that may disable performance of large-scale screenings, are not excluded. Such adverse effects, however, can be eliminated by introduction of internal and external standards. As an internal standard, we used GST-cJun *in vitro* phosphorylated by JNK2 (Forrer *et al.*, 1998). Alternatively, a JNK enzyme sample with defined activity or a lyophilised extract from activated NIH3T3 cells could be used as external standards.

In conclusion, we established a colorimetric ELISA-based JNK assay that could assist in monitoring of JNK activity in cell extract samples of different origin. This novel procedure represents a marked improvement upon conventional radioactive JNK assays in terms of sensitivity, accuracy, low time consumption, high throughput, and amenability to automation. To our knowledge, this is the first reported ELISA-based kinase assay that specifically measures a single selected kinase activity in cell extracts. It has a potential to be adapted for other kinase-substrate pairs where the interactions are sufficiently specific and where the phosphoepitope-specific antibodies are available.

Materials and Methods

Materials

The following reagents were used: polystyrene 96-well microtiter plates Immulon 4 (Dynatech Laboratories, Embrach-Embraport, Switzerland); Ni-NTA agarose and the pQE30 system (Qiagen, Basel, Switzerland); glutathione Sepharose 4B (Amersham Pharmacia Biotech, Dübendorf, Switzerland); polyclonal antibodies detecting c-Jun phosphorylated on Ser73, and ATF2 phosphorylated on Thr71 (New England Biolabs, Beverly, MA, USA); polyclonal antibody detecting JNK1 (C-17) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Complete Mini EDTA-free protease inhibitor cocktail and 4-nitrophenylphosphate (4-NPP) (Boehringer Mannheim, Mannheim, Germany); polyethylene glycol (PEG) 8000 (Fluka, Buchs, Switzerland); bovine serum albumin (BSA) (Sigma, Buchs, Switzerland); and Dulbecco's Modified Eagle Medium (DMEM) and donor calf serum (DCS) (Life Technologies, Basel, Switzerland). All other reagents were of the highest purity available.

Bacterial Expression Vectors

Bacterial expression vectors (pGEX derivatives, Pharmacia Biotech) for GST-cJun (aa 1–79) and GST-ΔcJun (aa 43–223) (Derijard *et al.*, 1994), GST-ATF2 (aa 1–109) and GST-ΔATF2 (aa 60–109) (Gupta *et al.*, 1995) were kindly provided by Dr. R. Davis (University of Massachusetts Medical School, Worcester, MA, USA). Plasmids for the expression of monomeric protein substrates were generated by employing the novel bacterial expression vector pDEX (Forrer and Jaussi, 1998), which is a pQE30 derivative containing the bacteriophage lambda coat protein D (pD) as fusion partner. The appropriate DNA fragments were PCR amplified using the above mentioned GST expression vectors as templates, followed by subcloning into pDEX. Expression vectors for the following N-terminally His_6-tagged pD fusion protein substrates were constructed: pD-cJun (aa 1–79), pD-ΔcJun (aa 43–79), pD-ATF2 (aa 1–99), and pD-ΔATF2 (aa 60–99). JNK1α1 and JNK2α2 (Kallunki *et al.*, 1994; Gupta *et al.*, 1996) were PCR amplified from the plasmids pcDNA3-JNK1 and pcDNA3-JNK2 (kindly provided by Dr. B. Dérijard; Centre de Biochimie, Nice, France) and subcloned into pQE30. A bacterial expression vector for His_6-tagged p38α (Han *et al.*, 1994) was kindly provided by Dr. J. Han (The Scripps Research Institute, La Jolla, CA, USA). Bacteriophage lambda protein phosphatase (lambda PPase) was PCR amplified using wild-type phage and subcloned into pQE30.

Recombinant Proteins

The GST fusion proteins were expressed in *E. coli* and purified by glutathione affinity chromatography according to the instructions from Pharmacia Biotech. All His_6-tagged proteins were expressed in *E. coli* and purified by Ni^{2+}-chelate chromatography according to the instructions from Qiagen with the exception of lambda PPase where all buffers and the bacterial culture media were supplemented with 4 mM $MnCl_2$. All purified proteins were dialysed against Tris buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and stored at −70 °C in aliquots. Protein concentrations were estimated by a modified Lowry method using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Glattbrugg, Switzerland) and BSA as a protein standard.

Cell Culture and Preparation of Cell Extracts

NIH3T3 cells were maintained in DMEM supplemented with 10% donor calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Extract from UV-treated cells was prepared by starving the cells for 20 h by reduction of serum to 0.2%, followed by UV-C irradiation with 200 J/m^2 using the Stratalinker UV-crosslinker (Stratagene), and incubation for 1 h. The cells from a 150 cm^2 culture plate were then rinsed with ice cold phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl), harvested with a rubber policeman in 1 ml ice cold PBS containing 1 mM Na_3VO_4 and 25 mM NaF, collected by short spin centrifugation, resuspended in 0.5 ml NP-40 lysis buffer [LB; PBS containing 0.1% (w/v) Nonidet P-40, 25 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM EGTA, 1 tablet/10 ml of Complete Mini EDTA-free], incubated on ice for 30 min, and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was retained and the protein concentration was estimated using the Bio-Rad DC protein assay.

JNK Assay (ELISA)

The microtiter plate was coated with 50 μl/well of GST-cJun protein substrate solution (10 μg/ml in TBS) for 2 h at 37 °C in a microtiter plate incubator (Solo, Denley, Billingshurst, England). After washing four times (unless otherwise specified, all washes were performed by rinsing with deionised water), the plate was incubated with 200 μl/well of blocking buffer (BB; TBS containing 0.25% BSA, 0.05% Tween-20, and 0.02% NaN_3) for 30 min at room temperature. The plate was then washed twice, vortexed for 10 s, washed two times, and tempered at 4 °C for 10 min. Subsequently, 50 μl/well of cell extract in LB were loaded and incubated overnight (approximately 12 h), or for 3 h if PEG was added, at 4 °C. The plate was then rapidly washed twice with 200 μl/well TBS_{500} (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.05% Tween-20, and twice by adding TBS_{500} without detergent for 5 min at 37 °C, with vortexing after all wash steps. The kinase reaction was initiated by adding 50 μl/well of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 100 μg/ml BSA, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM ATP) and allowed to proceed for 1 h at 37 °C. The plate was then washed three times before it was incubated with phosphoepitope-specific antibody (1:2000 in BB, 50 μl/well) for 1 h at 37 °C. After three washes, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000 in BB, 50 μl/well) was added for 1 h at 37 °C to capture the antibody-phosphorylated protein substrate complex. Finally, the plate was washed three times, then washed and vortexed twice, and 75 μl/well of alkaline phosphatase substrate solution (3 mM 4-NPP, 50 mM $NaHCO_3$, 50 mM $MgCl_2$) were added for 1 to 4 h at 37 °C. The formation of 4-nitrophenolate was measured at 410 nm using a microtiter plate reader (Dynatech MR4000, Dynatech Laboratories). The detection was linear until not more than about 4% of the plastic-bound substrate was phosphorylated as deduced from signals generated by fully phosphorylated GST-cJun.

Competition Assay

First, the recombinant JNK1α1, JNK2α2, and p38α were dephosphorylated by lambda PPase to ensure the inactivation of partially autophosphorylated enzymes. Kinase solutions (1 mg/ml in TBS) were supplemented with 4 mM $MnCl_2$ and 10 μg/ml lambda PPase, and incubated for 1 h at 37 °C. The decline of kinase activity was monitored as previously described (Forrer *et al.*, 1998). The kinase solutions were diluted two times, and for competition, 50 μl/well of dephosphorylated kinases were allowed to pre-bind in the GST-cJun coated microtiter plate for 12 h at 4 °C. Subsequently, 50 μl/well of cell extracts in modified LB [TBS containing 0.1% (w/v) Nonidet P-40, 50 mM NaF, 2 mM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM EGTA, 2 tablets/10 ml Complete Mini EDTA-free] were added, and incubated for additional 12 h at 4 °C.

All further steps corresponded to the protocol described in the preceding paragraph.

For the competition by recombinant GST and GST-cJun, the extracts from UV-treated NIH3T3 cells (1 mg/ml) were supplemented with recombinant GST and GST-cJun to a final concentration of 2, 20 and 200 μg/ml and incubated on ice for 2 h. Subsequently, 50 μl/ml of the mixtures were incubated on GST-cJun coated microtiter plate overnight at 4 °C, and ELISA was performed as described above.

Acknowledgements

We thank R.J. Davis, B. Dérijard, and J. Han for the gift of various plasmids; and J. Jiricny for comments on the manuscript. This work was partially supported by the Bundesamt für Bildung und Wissenschaft, EU grant # FI4P-CT96-0043, BBW # 96.0123. R.T. was supported by the Hauptabteilung für die Sicherheit der Kernanlagen (HSK), contract # 63665.

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Received January 4, 1999; accepted March 8, 1999