Dual Specificity of the Interleukin 1- and Tumor Necrosis Factor-activated β Casein Kinase*

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François Guesdon‡, C. Graham Knight§, Lesley M. Rawlinson¶, and Jeremy Saklatvala¶

From the ¹Kennedy Institute of Rheumatology, Hammersmith, London, W6 8LH, United Kingdom, the §Department of Cell Adhesion and Signalling, Strangeways Research Laboratory, Cambridge, CB1 4RN, United Kingdom, and the Division of Molecular and Genetic Medicine, Royal Hallamshire Hospital, Sheffield, S10 2JF, United Kingdom

Tumor necrosis factor (TNF) and interleukin 1 (IL1) activate a protein kinase, TIP kinase, which phosphorylates β casein in vitro. We have now identified its main phosphorylation site on β casein, Ser$^{124}$ ($K_m \approx 28 \mu M$), and a minor phosphorylation site, Ser$^{142}$ ($K_m \approx 0.7 \mu M$). The sequence motif that determined the phosphorylation of Ser$^{124}$ by the kinase was studied with synthetic peptides bearing deletions or substitutions of the neighboring residues. This allowed synthesis of improved substrates ($K_m \approx 6 \mu M$) and showed that efficient phosphorylation of Ser$^{124}$ was favored by the presence of large hydrophobic residues at positions +1, +9, +11, and +13 (counted relative to the position of the phosphoacceptor amino acid) and of a cysteine at position −2. Peptides in which Ser$^{124}$ was replaced by tyrosine were also phosphorylated by TIP kinase, showing it to have dual specificity. It is unable to phosphorylate the MAP kinases in vitro and is therefore not directly involved in their activation. Its biochemical characteristics indicate that TIP kinase is a novel dual specificity kinase, perhaps related to the mixed lineage kinases. It copurified with a phosphoprotein of about 95 kDa, which could correspond either to the autophosphorylated kinase or to an associated substrate.

The inflammatory cytokines interleukin 1 (IL1) and tumor necrosis factor (TNF) are structurally unrelated and bind to distinct receptors, but have similar biological effects. Each can activate endothelial cells and cause leukocyte accumulation, tissue destruction, fever, and the acute phase response (1).

The intracellular mechanisms of action of IL1 and TNF are incompletely known, but appear to rely heavily on protein phosphorylation (2, 3). The two cytokines are known to induce tissue destruction, fever, and the acute phase response (1). Tumor necrosis factor (TNF) is structurally unrelated and binds to the TNF receptors are associated with intracellular pathways, or cytokines other than IL1 and TNF (26). To refer to this highly specific regulation and to avoid the confusing term “casein kinase,” it has recently been renamed TNF/IL1-activated protein kinase (TIP) kinase (27).

TIP kinase is a cytosolic enzyme of about 100 kDa that may be part of larger aggregates of 200–600 kDa in vivo (10, 26, 27). Its physiological substrates are unknown. Unlike the components of the MAPK cascades, it is not inactivated by phosphoprotein phosphatases in vitro and therefore appears to be regulated by a mechanism other than direct phosphorylation, perhaps via association with other cytosolic proteins (26). We have now identified the main phosphorylation site of TIP kinase on β casein and have investigated the role of neighboring residues in the reaction. Our findings indicate that TIP kinase corresponds to a new type of dual specificity protein kinase, directed toward a novel sequence motif.

MATERIALS AND METHODS

Reagents—Human recombinant IL1α and TNF-α expressed in Escherichia coli were purified as described previously (27). Baculovirus-expressed recombinant human cytosolic phospholipase A$_2$ and iBET$_2$ proteins were kind gifts from Dr. Graham Ritchie (Zeneca Pharmaceuticals, Macclesfield, UK) and Dr. Martha Ladner (Chiron Corporation, Emeryville, CA), respectively. ATF2, p42 MAPK, p54 MAPK, p38 MAPK, MKK1, and MKK4 were expressed as glutathione S-transferase fusion proteins in E. coli and purified by standard methods. The cDNA encoding human MKK4 was generously given by Dr. L. B. Holzman (Division of Nephrology, University of Michigan Medical School, Ann Arbor, MI); that for rat p54 MAPK (stress-activated protein kinase β) by Dr. James R. Woodgett (Ontario Cancer Institute, Princess Margaret
Hospital, Toronto, Ontario, Canada); those for human MKII and p42 MAPK by Professor Christopher Marshall (Institute for Cancer Research, Chester Beatty Laboratories, London, UK); and those for human ATF2 and p38 MAPK by Simon Lamb (Celltech Ltd, Slough, UK). The antisera to the 225 carbonyl-terminal amino acids of dual leucine zipped receptor kinases (DLK) was a generous gift from Dr. Holzman. The antibodies to peptides corresponding to residues 828–847 of mixed lineage kinase (MLK) 3 and residues 589–608 of receptor interacting protein (RIP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Molecular mass standard proteins, β casein, and ATP were from Sigma (Poole, UK). [γ−32P]ATP was from Amersham International (Little Chalfont, UK). Polybrene (IBA) was from Aldrich Chemical Corporation, Milwaukee, WI, N-0-Fluoromethylmethoxycarbonyl (Fmoc)–amino acids were from Calbiochem-Novabiochem (Beeston, UK).

Substrate Peptides—The proteolytic fragment of β casein, T1, was prepared by digestion of the protein with 1-1-losylaminol-2-phenylethylimidomethyl ketone-treated trypsin (Sigma), precipitation at pH 4.6, and anion exchange chromatography on Mono Q (Pharmacia-Biotech, Saint Albans, UK) as described previously (27). Synthetic peptides were made on peptide amide linker-polyethylene glycol-polyestrene resin (PerSeptive Biosystems, Hertford, UK) by the Fmoc method (29), with activation by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (PerSeptive Biosystems) (30). Syntheses were carried out either in a 9050 Plus automatic peptide synthesizer (PerSeptive Biosystems) or manually in a multipeptide synthesizer (Cambridge Research Biochemicals, Northwich, UK). The peptides were purified by reverse-phase chromatography. Their purities and molecular weights were confirmed by analytical reverse-phase high pressure liquid chromatography and mass spectrometry (27).

Cells—MRC5 fibroblasts from the National Institute for Biological Standards and Control (Potters Bar, UK) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Paisley, UK) containing 160 mg/ml crystapen, 100 mg/liter streptomycin, and 10% heat-inactivated fetal calf serum.

Preparation of TIP Kinase—Cytosols of IL1-stimulated fibroblasts were chromatographed on the anion exchanger Resource Q (Pharmacia Biotech Inc.) as in Guesdon et al. (26), and fractions containing the kinase were pooled. The enzyme was then precipitated by addition of 0.33 volume of a chilled saturated solution of (NH4)2SO4. After a 30-min incubation on ice, the suspension was spun at 13,000 × g for 10 min. The pellet was usually resuspended in 0.25 volume of Resource Q chromatography buffer (26) supplemented with 200 mM NaCl, 10% glycerol, and 0.1% Brij 35. In some experiments, the ammonium sulfate precipitates were resuspended in 0.05 volume (120 μl) of gel filtration buffer (Resource Q buffer supplemented with 250 mM NaCl and 0.05% Brij 35, v/v) and chromatographed on a Superose 12 HR 10/30 column in the same buffer at 0.3 ml/min (26). Fractions of 0.25 ml were collected. Control preparations to assess the activity of contaminating kinases were made by applying the same purification procedure to cytosols of unstimulated cells (10, 26). In Vitro Phosphorylations—These were done at room temperature in 20-μl reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 40 μM ATP, and 15 kBq/μl [γ−32P]ATP, except when labeling substrates for mapping, for which the ATP concentration was 1 μM (27). Reactions were stopped after 20–50 min by addition of twice-concentrated electrophoresis sample buffer (27) at 100 °C, and the mixtures were resolved by gel electrophoresis.

Gel Electrophoresis—Electrophoresis of proteins (SDS-polyacrylamide gel electrophoresis) was done on slab gels cast with a mixture of acrylamide and N,N-methylenebis-acrylamide (38:1 by weight). Acrylamide concentrations were 5% for electrophoresis of a phosphoprotein of about 95 kDa, called pp95, 10.5% for other proteins and 16% for IBA fragments of β casein (w/v). The gels were stained with Coomassie Blue and dried before autoradiography. Electrophoresis of CNBr fragments and synthetic peptides was done on area-containing gels as described elsewhere (27).

Mapping of β Casein—Radiolabeled β casein was extracted from gel slices and precipitated with trichloroacetic acid as in Guesdon et al. (10). Cleavage by IBA (1 μg/ml) was carried out following published methods (31, 32), β casein being at 1 μg/ml. The digests were desalted by gel filtration on columns of Sephadex G25 (Pharmacia). Cleavage by CNBr (1 μg/ml) was done in 70% formic acid and 1 mM dithiothreitol for 24 h; β casein was at 20 mg/ml. The reaction mixtures were then diluted 10 times in water, freeze-dried, and subjected to analytical high pressure liquid chromatography (27) on a Tephrocop 10C18 column (HPLC Technology, Macclesfield, UK). Elution was by 25-ml linear gradients of 5 to 80% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min (27). For identification of the fragments, desalted digests or chromatography fractions were subjected to gel electrophoresis. The fragments were then electrotransferred to polyvinylidene difluoride membrane, detected by Coomassie Blue staining or autoradiography and subjected to NH2-terminal sequencing as described previously (27).

Kinetic Studies—Phosphorylations were quantified by counting the Cerenkov radiation from pieces of gels containing the substrates. Background phosphorylation by constitutively active contaminating kinases was measured in assays carried out with the control kinase preparation from unstimulated cells. Counts obtained with the TIP kinase preparation were corrected for background and fitted to the Michaelis-Menten equation by non-linear regression with the program EnzFitter (33).

RESULTS

Search for Physiological Substrates of TIP Kinase—We started our investigation of the substrate specificity of TIP kinase by testing whether or not it could phosphorylate several proteins known to be involved in responses to IL1 and TNF, such as cytosolic phospholipase A2, the transcription regulators c-Jun, IκBα, and ATF2, the protein kinases p42 MAPK, p54 MAPK, p38 MAPK, and the activator of p42 MAPK, MKK1. The recombinant proteins and β casein were subjected to in vitro phosphorylation by preparations of TIP kinase and by control preparations, made by the same procedure from unstimulated cells. As previously reported (10, 26), the main product of the phosphorylation of β casein by TIP kinase was a phosphoform of the protein, called R, which was electrophoretically retarded relative to the unphosphorylated casein (Fig. 1). An unretarded phosphoform, called U, was also observed, but was less abundant than the R form and phosphorylations by control preparations indicated that it was produced, in part, by contaminating protein kinases (Fig. 1). The only other substrate protein that was phosphorylated by the TIP kinase preparation was the recombinant p38 MAPK, but its phosphorylation was very weak and was also observed when control kinase preparations from unstimulated cells were used (Fig. 1).

The phosphorylation of p38 was thus due to a contaminating kinase, and none of the proteins tested were substrates of the IL1-activated enzyme. Other similar experiments showed that TIP kinase had no activity on the activator of p54 MAPK, MKK4, or on hsp27, myelin basic protein, phosvitin, or histone H1 (data not shown). We also attempted to identify substrates...
terminal sequences are given.

Labeled fragments are shown as black horizontal bars. Their amino-terminal sequences confirmed that complete under the conditions used (Fig. 2). The masses and phoacceptor amino acid).

Identification of the Main Phosphorylation Site on β Casein—The main phosphorylation sites of TIP kinase have previously been shown to be serines located in a 6.4-kDa tryptic fragment of β casein, called T1 (27). The fragment, which is phosphorylated with kinetics similar to those of the whole protein (K_m = 27 ± 6 μM), corresponds to residues 114–169 and contains seven serines (27, 31). To locate the main phosphorylation site more precisely, the R phosphoform of β casein was subjected to further mapping after cleavage by IBA and CNBr.

Treatment of the R phosphoform with IBA generated two fragments of molecular masses 24 and 6 kDa; some intact β casein was also observed, indicating that cleavage was not complete under the conditions used (Fig. 2A). The masses and amino-terminal sequences of the fragments confirmed that they resulted from cleavage of the protein on the carboxyl side of its single tryptophan, Trp143, in agreement with previous findings (31). Only the 24-kDa amino-terminal fragment was labeled, indicating that phosphorylation was on the amino side of Trp143 (Fig. 2, A and C). Cleavage of R phosphoform by CNBr generated a complex mixture of fragments which was fractionated by reverse-phase chromatography as described under “Materials and Methods.” The digest was treated with CNBr and the digest was fractionated by reverse-phase chromatography as described under “Materials and Methods.” The curves show the best fits of the data to the Michaelis-Menten equation. The computed K_m values were 24 (T1), 37 (P1), and 4.5 μM (C122) in this experiment. The corresponding V_max values were 2.4 (T1), 2.4 (P1) and 2.6 nmol·min⁻¹·μM⁻¹ (C122).

To discriminate between the three candidates, we then tested the phosphorylation of synthetic peptides mimicking these regions of the β casein sequence. The first two peptides, called P1 and P2, corresponded, respectively, to residues 116–141 and 129–155 of β casein. Peptide P1 was phosphorylated by TIP kinase with kinetics similar to those measured with tryptic fragment T1 (Fig. 3). P2 was also phosphorylated, although much less efficiently than P1 (Table I). This indicated that the main phosphorylation site had to be either Ser122 or Ser124 and that Ser142 corresponded perhaps to a minor phosphorylation site. Variants of P1 in which the candidate phoacceptors were replaced by the non phosphorylatable residue, cysteine, were then tested. TIP kinase was unable to phosphorylate the variant peptide C124, in which Ser124 was replaced by cysteine, but phosphorylated peptide C122, in which Ser122 had been replaced by cysteine (Fig. 3). This showed that Ser124 was the main phosphorylation site of TIP kinase on β casein and that Ser122 was not a target of the enzyme. Interestingly, kinetic analysis of the phosphorylation of C122 showed a 7-fold decrease in K_m value, relative to that of P1, with no change of V_max (Fig. 3, Table I), indicating that cysteine was strongly preferred to serine at position –2 (residue numbers are counted relative to the position of the phoacceptor amino acid).

Dependence of Ser124 Phosphorylation upon Peptide Length—To define the boundaries of the sequence motif recognized by TIP kinase, we synthesized a series of progressively shorter variants of P1, called D1 to D5, and compared their phosphorylations to that of the parent peptide. We avoided replacing Ser122 by Cys for synthetic convenience. Removal of

atated by HPLC. The 32P-labeled material eluted as a peak of retention time 14.5 min whose analysis by electrophoresis showed a single labeled fragment (Fig. 2B). Its amino-terminal sequence, PFPKY, identified its amino-terminal residue as Pro110 (Fig. 2B). Its carboxyl-terminal residue was likely to be the next methionine in the sequence of β casein, Met144 (31). These results placed the phosphorylation site between Pro110 and Trp143, in a region that contained three serines at positions 122, 124, and 142 (Fig. 2C).

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Dependence of Ser124 Phosphorylation upon Peptide Length—To define the boundaries of the sequence motif recognized by TIP kinase, we synthesized a series of progressively shorter variants of P1, called D1 to D5, and compared their phosphorylations to that of the parent peptide. We avoided replacing Ser122 by Cys for synthetic convenience. Removal of
two or four residues of the carboxyl end of P1 had no significant
effect on the kinetics of phosphorylation (peptides D1 and D2,
Table II), indicating that the residues on the carboxyl side of
Leu137 were not required for phosphorylation of Ser124. Further
shortening of the carboxyl-terminal end by two residues re-
sulted in an increase in Km of about 10-fold and a commensu-
rate decrease in Vmax/Km (peptide D3, Table II). Peptide D4,
which had an even shorter carboxyl end, was not phosphoryl-
ated by TIP kinase (Table II). These results suggested that
some of the residues located between Asn132 (position
2) and Pro139 (position +14) played an important role in the recog-
nition of Ser124 by TIP kinase. Removal of four residues at the
amino end of P1 increased Km by 50%, indicating that the residues
on the amino side of Thr120 played only a minor role in the
reaction (peptide D5, Table II).

Alanine Scanning of Peptide D5—A set of variants of peptide
D5 in which residues Thr120 to Leu137 were systematically
replaced by alamines (except for Ser124) was then synthesized,
and the effects of the substitutions on the kinetics of phosphy-
loration were investigated. The most dramatic effect was
caused by the substitution of Leu125 (position +1), which in-
creased Km 30-fold (Fig. 4). Substitutions to alanine of Thr126,
Asp129, Leu133, Leu135, and Leu137 (positions
1, 5, 9, +11) also increased Km by 3–7-fold, and weaker effects
(2–3-fold increases in Km) were caused by substitutions of
Gln132 (position −1) and Val139 (position +6) (Fig. 4). Only one
substitution to alanine decreased the Km value, that of Ser122
(position −2); the decrease was by less than 2-fold, and was thus
not as dramatic as the 7-fold decrease observed when Thr120
was replaced by a cysteine. The other substitutions to
alanine had no significant effects on the kinetics of phosphy-
loration (Fig. 4).

As had been the case for the deletion variants D3 and D4, no
significant changes in Vmax values were caused by any of the
substitutions to alanine and only changes of Vmax/Km values
that were inversely proportional to the changes in Km were
observed (data not shown). These results showed that eight
residues of β casein, located at positions −1, +1, +2, +5, +6
+9, +11, and +13, were contributing to the reactivity of Ser124
toward TIP kinase. In particular, the requirement for the three
leucines at positions +9, +11, and +13 was in agreement with
the results observed earlier with the deletion variants D3 and
D4 (Table II).

Residue Preferences at Positions +1, +2, +5, +9, and +11—A preliminary characterization of the motif targeted by
TIP kinase was made by studying the effects of replacing the
residues at positions +1, +2, +5, +9, and +11 by amino acids
of varying charges or hydrophilicity. The parent peptide for
that experiment, E1 (sequence NEAQSLTATDVENLHLPL-
PLLQ-NH2), was a variant of D5 in which the residues at
positions −4, −2, and +3 had been replaced by Asn, Ala, and
Ala, respectively, to facilitate synthesis and improve solubility.
Its kinetics of phosphorylation were similar to those of D5
(Table II).

Substitution of Leu125 (position +1) by Phe in E1 caused a
small (2-fold) increase in Km, whereas its substitution by either
Asn, Asp, or Lys had very strong adverse effects (Fig. 5). These
results suggested a strict requirement for a large hydrophobic
amino acid (Leu or Phe) at position +1. The requirement at
positions +2 and +5 appeared less strict. Substitution of
Thr126 (position +2) by Asn caused no significant change to the
kinetics of phosphorylation, substitutions by Ile or Phe in-
creased the Km, thereby reducing its substrate specificity;
substitutions by Ser or Phe increased the Km, thereby reducing
the substrate specificity of TIP kinase at positions +4, +2, +5, +9, and +11.

Synthesis of Single Phosphoacceptor Substrates—The se-
quence surrounding Ser124 contains three threonine residues

| Peptide | Sequence | Km | Relative Vmax | Relative Vmax/Km |
|---------|----------|----|---------------|-----------------|
| P1      | VEPFTEQSLTLDVENLHLPLLQ-NH2 | 28 ± 7 | 1.1 ± 0.1 | 1.1 ± 0.2 |
| P2      | DVENLHLPLLQMAIQHOPQLPFTV-NH2 | 700 ± 300 | 0.41 ± 0.13 | 0.020 ± 0.003 |
| C122    | VEPFTEQSLTLDVENLHLPLLQ-NH2 | 4.2 ± 0.4 | 1.0 ± 0.1 | 6.8 ± 0.7 |

| Peptide | Sequence | Km | Relative Vmax | Relative Vmax/Km |
|---------|----------|----|---------------|-----------------|
| D1      | VEPFTEQSLTLDVENLHLPLL-NH2 | 31 ± 5 | 1.0 ± 0.1 | 1.0 ± 0.2 |
| D2      | VEPFTEQSLTLDVENLHLPLL-NH2 | 32 ± 2 | 0.83 ± 0.04 | 0.70 ± 0.04 |
| D3      | VEPFTEQSLTLDVENLHL-NH2 | 315 ± 51 | 0.72 ± 0.11 | 0.066 ± 0.006 |
| D4      | VEPFTEQSLTLDVE-NH2 | np^ | np | np |
| D5      | TESQSLTLDVENLHLPLLQ-NH2 | 47 ± 8 | 1.2 ± 0.1 | 1.0 ± 0.1 |
| E1      | NEAQSLTATDVENLHLPLLQ-NH2, | 46 ± 1 | 0.74 ± 0.25 | 0.57 ± 0.18 |
| E2      | VEPFTEQSLTLDVENLHLPLLQ-NH2 | 5.9 ± 0.9 | 1.9 ± 0.3 | 5.5 ± 2.2 |
| E3      | VEPFTEQSLTLDVENLHLPLLQ-NH2 | 6.8 ± 0.2 | 0.87 ± 0.25 | 3.5 ± 1.0 |

* np, no detectable phosphorylation.
that could constitute phosphorylation sites of other protein kinases, thus potentially contributing to background phosphorylation by contaminating enzymes in crude preparations of TIP kinase. In particular, the two threonines at positions +2 and +4 are possible targets of CK2 (28). The substitutions carried out on D5 and E1 had indicated that the threonines could be replaced by either asparagine or alanine with no adverse effect. We thus synthesized two variants of C122, called E2 and E3, in which Thr126 had been replaced by an asparagine and the two other threonines by either alanines or asparagines (Table II). E2 and E3 were phosphorylated with kinetics similar to those of C122 (Table II). Their efficient phosphorylation and absence of phosphorylatable residues other that the target serine made them very specific substrates of TIP kinase.

Phosphorylation of Tyrosine-containing Peptides—Variants of peptide D5 in which Ser124 was replaced by threonine or tyrosine, respectively called D5-T and D5-Y, were also synthesized and their phosphorylations were compared with that of D5. Interestingly, D5-T and D5-Y were both phosphorylated by the TIP kinase preparations (Fig. 6A). Phosphorylation of the tyrosine-containing variant was unexpected because most serine/threonine-specific protein kinases are unable to phosphorylate tyrosine residues (34). Phosphorylation of D5-Y by control preparations from unstimulated cells was much weaker than by preparations from IL1-stimulated cells, indicating that the D5-Y phosphorylating activity was stimulated by IL1. The magnitude of its stimulation was similar to that of TIP kinase, monitored with D5 and D5-T (Fig. 6A). This suggested that the D5-Y phosphorylating enzyme could correspond to TIP kinase itself, rather than being a contaminating enzyme, and that TIP kinase could be a dual specificity kinase.

The kinetics of phosphorylation of D5-Y by the IL1-activated tyrosine-kinase were similar to those of D5 and D5-T (Fig. 6B, Table III). The $K_m$ value for D5-Y phosphorylation was identical to that measured with D5, while that of D5-T was twice that value. Comparisons of the $V_{\text{max}}/K_m$ values of D5, D5-T, and D5-Y indicated that the efficiency of the phosphorylation of D5-Y was intermediate between those of D5 and D5-T (Tables 2 and 3). We also synthesized a variant of E3, called E3-Y, in which the phosphoacceptor serine was replaced by tyrosine. E3-Y was phosphorylated by the IL1-activated tyrosine kinase, but its phosphorylation was less efficient than that of D5-Y, with a 5-fold greater $K_m$ and a 10-fold smaller $V_{\text{max}}/K_m$ (Table...
ammonium sulfate (Fig. 6). The two activities also co-precipitated in the presence of 25% NaCl concentration of about 0.2M, which was earlier than with ammonium sulfate precipitation. The precipitates were chromatographed on Superose 12 as described under “Materials and Methods.” Fractions were assayed for activities phosphorylating either E3 at 100 μM (A) or E3-Y at 300 μM (B). The reaction mixtures were electrophoresed and the gels were autoradiographed. Strips of autoradiograms containing the substrates are shown. The fraction numbers and the elution positions of the molecular mass standard blue dextran (V0), apoferritin (443 kDa), yeast alcohol dehydrogenase (141 kDa), phosphorylase b (94 kDa), and transferrin (78 kDa) are shown. C, detection of pp95. Fractions 30–38 of the Superose 12 chromatograms assayed in panels A and B were subjected to in vitro phosphorylation in the absence of exogenous substrate. Autoradiograms of the gels are shown. The migration positions of the molecular mass standards β-galactosidase (116 kDa), phosphorylase b (97 kDa), and fructose-6-phosphate kinase (84 kDa) are indicated.

III). The decreased efficiency of phosphorylation of E3-Y, relative to D5-Y, was in contrast to the improved efficiency of phosphorylation of its serine-containing analogue, E3, relative to D5. The modifications of the β-casein sequence that had improved the kinetics of phosphorylation of E3 were thus adverse to the tyrosine-phosphorylating activity.

**Co-purification of the Tyrosine Phosphorylating Activity with TIP Kinase**—We then compared the chromatographic behavior of the IL1-activated tyrosine phosphorylating activity, assayed with peptide E3-Y, to that of TIP kinase, assayed with peptide E3. Assays of the Resource Q fractions from cytosols of resting and IL1-stimulated cells showed that the distributions of TIP kinase and the E3-Y kinase in fractions eluted from the high resolution anion exchanger were identical (data not shown). The two activities also co-purified during gel electrophoresis on a preparative gel filtration column (Fig. 7A). They both eluted in fractions 34–38 of the chromatogram, with maxima in fractions 35 and 36, which corresponded to an apparent molecular mass of about 500 kDa (Fig. 7A), in agreement with previous determinations of the apparent molecular mass of TIP kinase (22). This indicated that E3 and E3-Y were phosphorylated by the same oligomeric enzyme.

**Association of TIP Kinase with a 95-kDa Phosphoprotein**—The ability of TIP kinase to phosphorylate serine, threonine, and tyrosine residues and its other biochemical characteristics suggested that it could be related to the recently described family of MLKs, which have been proposed as possible dual specificity protein kinases on the basis of the sequences of their catalytic domains (35–37). We therefore looked for possible immunological similarities between TIP kinase and two MLKs for which antibodies were available, MLK3 and the DLK. Resource Q and Superose 12 fractions containing TIP kinase were subjected to Western blotting with these antibodies. Antigens of the expected sizes of MLK3 and DLK were detected in the Resource Q chromatograms. The DLK antigen eluted at an NaCl concentration of about 0.2 M, which was earlier than with TIP kinase (0.3 M NaCl). MLK3 eluted later than did DLK, but only partially overlapped with the elution position of TIP kinase activity (data not shown). Neither antigen was detected in the subsequent Superose 12 gel filtration fractions (data not shown). TIP kinase was thus distinct from the main MLK3 and DLK antigens. We also investigated whether or not it was immunologically related to the RIP, a 74-kDa protein kinase associated with the p55 TNF receptor whose sequence also suggests possible dual specificity (2, 5, 6). The antiserum to RIP that we tested detected no antigen in the TIP kinase-containing fractions (data not shown).

Because some MLKs have been shown to autophosphorylate in vitro, we also attempted to detect in vitro phosphorylation of endogenous proteins of the Superose 12 fractions. Interestingly the 95-kDa phosphoprotein pp95 was detected in the TIP kinase-containing fractions from IL1-stimulated cells, but not in those prepared from unstimulated cells. Like TIP kinase, it eluted from the gel filtration column in fractions 34–37, with highest amounts in fractions 35 and 36 (Fig. 7C). This indicated that pp95 was a component of 500-kDa complexes and was phosphorylated by an IL1-activated kinase. It could correspond either to the autophosphorylating TIP kinase, or to a distinct component of the oligomeric complexes. In agreement with the former hypothesis, the apparent molecular mass of pp95 on SDS-polyacrylamide gel electrophoresis gels was similar to that of a low molecular weight form of TIP kinase which had been detected in earlier studies and interpreted as being the monomeric enzyme (10, 26). Attempts at detecting TIP kinase activity after electrophoresis in substrate-impregnated gels by incubation of the gels in a buffer containing MgCl2 and [γ-32P]ATP were unsuccessful, and the phosphorylation of pp95 could not be detected in gels either (data not shown). Because of the loss of the peptide and pp95 phosphorylating activities during electrophoresis, it was not possible to determine whether or not TIP kinase corresponded to pp95.

**DISCUSSION**

We detected no phosphorylation of any protein other than β-casein by TIP kinase, either among the proteins involved in responses to IL1 that were tested individually, or in fractions of...
fibroblast cytosols. This suggested that TIP kinase had an unusual specificity and that its substrates were not major cytosolic proteins. The typical $K_m$ values of protein kinases for their substrates are between 0.5 and 20 $\mu$M (38–44). The $K_m$ value of TIP kinase for $\beta$ casein approaches the upper limit of this range (27), which suggested that some structural features of $\beta$ casein might resemble motifs present on the yet unknown physiological substrates of TIP kinase. We thus decided to investigate the determinants of the phosphorylation of $\beta$ casein. Mapping of the major phosphoform of the protein identified three candidate phosphoacceptors, the serines at positions 122, 124, and 142. Assays with synthetic peptides bearing each of the candidate serines then showed that Ser$^{124}$ was the main phosphorylation site and Ser$^{142}$, a minor site.

Peptides containing Ser$^{124}$ and extending to at least 13 residues on its carboxyl side were phosphorylated with kinetics identical to those of $\beta$ casein fragment T1 and the intact protein (27). This was a strong indication that TIP kinase was directed toward a sequence motif rather than a three-dimensional fold of native $\beta$ casein. Efficient phosphorylation of Ser$^{124}$ was primarily dependent upon the presence of hydrophobic residues at positions +1, +9, +11, and +13, of a cysteine residue at position −2 and, to a lesser extent, of an uncharged residue at position +2 and of a negatively charged residue at +5. No prolines or positively charged residues were needed. The variations to the catalytic efficiency caused by substitutions of residues other than the target serine were primarily due to variations of the $K_m$ parameter, and no significant changes to the $V_{max}$ value were observed. This indicated that the limiting step to the reaction was probably the binding of the peptides to the enzyme rather than catalysis. The residue preferences that we determined were thus favoring recognition of the peptides by the kinase, rather than speeding the turnover of the substrate-kinase complex.

The requirement of TIP kinase for a 16-residue long sequence motif in which most essential residues were neither charged nor prolines contrasts strongly with those of other protein kinases for which synthetic substrates are available. Typically, these are directed toward charged residues or prolines close to the phosphoacceptor residue (39–44). TIP kinase thus has an unusual substrate specificity. In particular, there is no resemblance to the substrate specificities of CK1 or CK2 (28, 43). This indicates that TIP kinase does not correspond to the CK1-like activity which has been found associated with the p75 TNF receptor (4). The unique substrate specificity of TIP kinase, combined with its exclusive activation by IL1 or TNF (26), implies that it probably mediates phosphorylations unique to the signaling mechanisms of the two inflammatory cytokines. This suggests a role for TIP kinase in controlling the specificity of the biological responses to IL1 and TNF, in contrast to the pathways leading to activations of the MAP kinases and NF-κB, which may be involved in more general responses.

The most interesting observation made in this study was that of the phosphorylation of peptides in which the target serine had been replaced by tyrosine. Most of the protein kinases that phosphorylate hydroxyl-bearing residues can be placed in one of two groups: those able to phosphorylate only the alcoholic groups of serine or threonine, and those phosphorylating their substrates only on the phenolic group of tyrosine (34). Only a few protein kinases, called dual-specificity kinases, are able to phosphorylate both types of hydroxyl-bearing residues (34, 45–49). Although we have not been able to purify TIP kinase to homogeneity, we obtained evidence which strongly suggests that it is responsible for the tyrosine phosphorylating activity. First, the activities phosphorylating the Ser$^{124}$-containing peptides and their Tyr$^{124}$-containing analogues were both strongly stimulated by IL1. Second, the stimulated serine and tyrosine phosphorylating activities were both cytosolic and co-purified during three purification steps. Third, the similar kinetics of phosphorylation of D5 and D5-Y indicated that the IL1-regulated tyrosine kinase activity was directed toward a sequence motif similar to that targeted by TIP kinase. The relatively high $K_m$ value measured with E3-Y was not contradictory to this interpretation, since much worse kinetics, with $K_m$ values in the millimolar range, would have been expected for D5-Y and E3-Y, had their phosphorylations being caused by a kinase directed toward a different motif (43, 44). These results strongly suggest that TIP kinase is a dual specificity kinase. The alternative interpretation, which we think is improbable, is that TIP kinase is a serine/threonine-specific protein kinase associated with a distinct IL1-activated tyrosine-specific kinase directed toward a similar sequence motif.

From the point of view of their structures, the known dual specificity kinases do not form a homogenous group but are instead distributed on several branches of the protein kinase family tree (34). One family of dual specificity kinases whose activation by IL1 or TNF is well documented is that of the MAPK activators, or MKks (11, 12, 20–22). Unlike TIP kinase, however, the MKks have molecular masses below 50 kDa and can be inactivated by phosphatases in vitro. TIP kinase has no activity on the three types of MAPK that were tested, but phosphorylates a variety of synthetic peptides based on the sequence surrounding Ser$^{124}$ of $\beta$ casein or, much less efficiently, peptides containing either Ser$^{57}$ (27) or Ser$^{142}$ of the protein. This is in contrast to the MKks for which no other substrates than the MAPks are known. TIP kinase is thus unlikely to be a relative of the MKks. The functions and characteristics of the other known dual specificity kinases indicate that they too are almost certainly not related to TIP kinase (34, 45–49). The available evidence suggests instead that two types of protein kinases, RIP and the MLks, could be cognates of TIP kinase.

Although there is not yet any evidence that they can phosphorylate tyrosine residues, both RIP and the MLks have been proposed as possible dual-specificity protein kinases on the basis of the sequences of their catalytic domains. RIP is a 74-kDa protein whose kinase domain resembles those of tyrosine kinases except for subdomains VI and VIII, which match the consensus sequences of Ser/Thr-kinases (2, 5). Conversely, the catalytic domains of the MLks have strong overall homology to that of the Ser/Thr-specific protein kinase Raf, but also contains short motifs normally diagnostic of tyrosine-phosphorylating activity in subdomains VI, IX, and XI (35). Their other characteristics suggest that the MLks are more likely to be related to TIP kinase than RIP. RIP interacts weakly with the p55 TNF receptor and mediates apoptosis, but has never been reported to be involved in IL1 signaling (2, 5, 6). The MLks resemble TIP kinase by their cytosolic location, their monomeric molecular masses (90–160 kDa) and their tendency to form oligomers of several hundred kilodaltons (35–37). Interestingly, recent evidence indicates that some MLks can function as upstream activators of the MKks, and thus have signaling functions (37, 50, 51). The antibodies to RIP, MLK3, and DLK that we tested did not react with TIP kinase, indicating that it is not closely related to RIP or to the two MLks. However, we cannot exclude a possible relationship of TIP kinase with other members of the MLk family. The antibodies that we used were directed against peptide sequences unique to either DLK or MLK3 and would not have cross-reacted with the other known MLks.

We also identified in TIP kinase-containing fractions the phosphoprotein pp95. In vitro phosphorylation of pp95 was
observed only in fractions from IL1-stimulated cells, which suggested that it could correspond either to TIP kinase itself or to a co-purifying substrate. Sequence data on pp95 will be necessary to determine whether or not it corresponds to TIP kinase and is related to the MLKs. Unfortunately, we could not obtain enough material for sequencing from fibroblasts.

An important bias of this study is its dependence upon a single phosphorylation site of a nonphysiological substrate. The alanine scanning of the Ser124 region could identify only a single phosphorylation site of a nonphysiological substrate. It would be interesting to determine if variations to the consensus motif existing in physiological substrates. Better phosphorylation of either tyrosine or serine/threonine, as suggested by the variations in catalytic efficiency observed with peptides D5, D5-Y, E3, and E3-Y. These future investigations would be greatly facilitated by using a combinatorial approach. Because it will screen a huge number of variants, such an approach should circumvent the bias inherent to the study of a casein-derived sequence.

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11. Saklatvala, J., Rawlinson, L. M., Marshall, C. J., and Kracht, M. (1993) FEBS Lett. 324, 189–192
12. Winston, B. A., Lange-Carter, C. A., Gardner, A. M., Johnson, G. L., and Riches, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1614–1618
13. Kyrakis, J. M., Banerjee, F., Nikolakaki, E., Tai, T., Ruhne, E. A., Ahmad, M. F., Avrush, J., and Woodgett, J. R. (1994) Nature 369, 156–160
14. Kracht, M., Traugó, O., Tot té, N., F., Shiroo, M., and Saklatvala, J. (1994) J. Exp. Med. 180, 2017–2025
15. Bird, T. A., Kyrakis, J. M., Tyszler, L., Gayle, M., Milne, A., and Virca, G. D. (1994) J. Biol. Chem. 269, 31836–31844
16. Freshney, N. W., Rawlinson, L. M., Guedson, P., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) Cell 78, 1039–1049
17. Ridley, S. H., Sarsfield, S. J., Lee, J. C., Bigg, H. F., Cawston, T. E., Taylor, D. J., DeWitt, D. L., and Saklatvala, J. (1997) J. Immunol. 158, 3165–3173
18. Rinaudo, G., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
19. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) FEBS Lett. 364, 229–233
20. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avrush, J., Kyrakis, J. M., and Zon, L. I. (1994) Nature 370, 784–788
21. Derijard, B., Rinaudo, G., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–684
22. Cuenda, A., Alonso, G., Morris, N., Jones, M., Meier, R., Cohen, P., and Nebreda, A. R. (1996) EMBO J. 15, 4156–4164
23. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Cell 84, 853–862
24. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
25. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Liu, X.-H., Basu, S., McGingley, M., Chan-Hui, P. Y., Lichenstein, H., and Kolesnick, R. (1997) Cell 89, 63–72
26. Guedson, F., Waller, R. J., and Saklatvala, J. (1994) Biochem. J. 304, 761–768
27. Guedson, F., Waller, R. J., and Saklatvala, J. (1997) Cytokine 9, 471–479
28. Pinna, L. (1998) Biochem. Biophys. Acta 1054, 267–284
29. Fields, G. B., and Noble, R. (1997) Int. J. Pept. Protein Res. 35, 161–214
30. Cargino, L. A., El-Faham, A., Minor, C. A., and Albericci, F. (1994) J. Chem. Soc. Chem. Commun. 201–203
31. Carless, C., Huet, J.-C., and Ribadeau-Dumas, B. (1988) FEBS Lett. 229, 265–272
32. Mahoney, W. C., Smith, P. K., and Hermosdon, M. A. (1981) Biochemistry 20, 443–448
33. Leatherbarrow, R. J. (1990) Anal. Biochem. 184, 274–278
34. Hanks, S. K., and Hunter, T. (1995) FASEB J. 9, 576–577
35. Duron, D. S., Devereux, L., Dietzsch, E., and de Kretser, T. (1993) Eur. J. Biochem. 213, 701–710
36. Gallo, K. A., Mark, M. R., Scadden, D. T., Wang, Z., Gu, Q., and Godowski, P. J. (1994) J. Biol. Chem. 269, 15092–15100
37. Pan, G., Merritt, B. K., Kortenmann, M., Shaw, P. E., and Holzman, L. B. (1996) J. Biol. Chem. 271, 24788–24793
38. Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987) Annu. Rev. Biochem. 56, 567–613
39. Force, T., Bonventre, J. V., Heidecker, G., Rapp, U., Avrush, J., and Kyrakis, J. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1270–1274
40. Stokoe, D., Caudwell, B., Cohen, P. T. W., and Cohen, P. (1993) Biochem. J. 296, 834–849
41. Leighton, I. A., Dalby, K. N., Caudwell, F. B., Cohen, P. T., and Cohen, P. (1995) FEBS Lett. 375, 289–293
42. Joseph, C. K., Byun H.-S., Bittman, R., and Kolesnick, R. A. (1995) J. Biol. Chem. 268, 20002–20006
43. Kemp, B. E., and Pearson, R. B. (1990) Trends Biochem. Sci. 15, 342–346
44. Kemp, B. E., Parker, M. W., Hu, S., Tiganis, T., and House, C. (1994) Trends Biochem. Sci. 19, 440–444
45. Haystead, T. A. J., Dent, P., Wu, J., Haystead, C. M., Sturgill T. W. (1992) FEBS Lett. 306, 17–22
46. Coleman, T. E., Tang, Z., and Dunphy, W. G. (1993) Cell 72, 919–929
47. Wang, Q. M., Fuo, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) J. Biol. Chem. 269, 14566–14574
48. Lanzé, E., Stoelcker, B., Laca, F. C., Weiss, E., Schultz, A. R., and Winey, M. (1995) EMBO J. 14, 1655–1663
49. Lawler, S., Fong, X.-H., Chen, H.-R., Marvoka, E. M., Turk, C. W., Griwsword-Prenner, I., and Derynick, R. (1997) J. Biol. Chem. 272, 14450–14455
50. Hirai, S.-I., Izawa, M., Osada, S.-I., Sproug, G., and Ohno, S. (1996) Oncogene 12, 641–650
51. Teramoto, H., Csoo, O. A., Miyata, H., Isgishi, T., Miki, T., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 27252–27228