The Intermediate Filament Protein Keratin 8 Is a Novel Cytoplasmic Substrate for c-Jun N-terminal Kinase*

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Keratins 8 (K8) and 18 are the primary intermediate filaments of simple epithelia. Phosphorylation of keratins at specific sites affects their organization, assembly dynamics, and their interaction with signaling molecules. A number of keratin in vitro and in vivo phosphorylation sites have been identified. One example is K8 Ser-73, which has been implicated as an important phosphorylation site during mitosis, cell stress, and apoptosis. We show that K8 is strongly phosphorylated on Ser-73 upon stimulation of the pro-apoptotic cytokine receptor Fas/CD95/Apo-1 in HT-29 cells. Kinase assays showed that c-Jun N-terminal kinase (JNK) was also activated with activation kinetics corresponding to that of K8 phosphorylation. Furthermore, K8 was also phosphorylated on Ser-73 by JNK in vitro, yielding similar phosphopeptide maps as the in vivo phosphorylated material. In addition, co-immunoprecipitation studies revealed that part of JNK is associated with K8 in vivo, correlating with decreased ability of JNK to phosphorylate the endogenous c-Jun. Taken together, K8 is a new cytoplasmic target for JNK in Fas receptor-mediated signaling. The functional significance of this phosphorylation could relate to regulation of JNK signaling and/or regulation of keratin dynamics.

Keratins constitute the largest group of the intermediate filament (IF)1 protein family. Their expression is strictly tissue-specific; for example, keratins 8 and 18 (K8/18) are preferentially expressed in simple epithelia. Phosphorylation of keratins at specific sites affects their functional and assembly state and has been suggested to play a role in cell signaling (1). Phosphorylation of the N-terminal Ser-33 on K18 enables a cell cycle-dependent interaction of K8/18 IFs with members of the 14-3-3 protein family, promotes depolymerization of K8/18 in vitro (2), and plays a role in the intracellular distribution of K8/18 polymers (3). K8 is also phosphorylated upon activation of the epidermal growth factor receptor (4) and by pro-urokinase stimulation (5). Several studies have shown that K8/18 phosphorylation is elevated upon cell stress (6, 7). Drug-induced hepatotoxic stress, induced by feeding mice with a giseofulvin-supplemented diet, results in a dramatic K8/18 hyperphosphorylation (7). Drug-induced apoptosis in epithelial cells leads to phosphorylation of K18 on Ser-52 and a marked reorganization of K8/18. Pervanadate-mediated tyrosine phosphorylation of K8 and -19 in a p38 kinase-dependent pathway has been reported recently (8). In cultured HT-29 cells, hyperphosphorylation of K8 at Ser-73 was associated with apoptosis induced by anisomycin or etoposide. The kinase regulating this site has not been identified, but members of the proline-directed mitogen-activated protein kinase (MAPK) family have been suggested as candidates (9). Because Ser-73 is phosphorylated upon various stress conditions, it could be a putative target for the stress-activated MAPK family members.

The MAPK family is composed of the prototype kinase, the mitogen-activated MAPK/ERK, and the stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 kinase. Both JNK and p38 kinase are involved in cellular responses to physical stresses, inflammatory cytokines, and apoptosis (10–13), whereas ERK is activated by growth factors and other mitogens. Among the most specific and physiological activators of JNK and p38 kinase are the death receptors, including the Fas receptor (FasR) and tumor necrosis factor receptor (13). The FasR, belonging to the TNF receptor family, is a physiological activator of apoptosis in a wide range of cell types. A number of reports have shown that JNK is an integral part of FasR-mediated signaling, both when cells are sensitive and insensitive to FasR-mediated apoptosis (14, 15). JNK can be elicited through many of the following mechanisms in response to FasR activation: through the Daxx protein (16); by caspase-dependent activation of MEKK1, a JNK kinase (17, 18); and by augmented levels of ceramide (20). In some reports and cell models (19, 20, 21), activation of JNK has been suggested to be independent of apoptosis, whereas in some
other models, FasR-mediated apoptosis seems to require JNK activity.

JNK was first identified as a kinase specific for phosphorylation of the transcription factor c-Jun (22). Since then, a number of other substrates, predominantly transcriptional factors, have been established. These include Elk-1, ATF, p53, DPC4, NFAT4, and Sap-1 (23, 24). However, very little is known about the possible cytoplasmic targets for JNK. In this respect, the neuronal intermediate filament neurofilament-H (NF-H) has been implicated as a JNK substrate, with phosphorylation at repeated Lys-Ser-Pro-X-Glu motifs within the C-terminal domain (25).

In this study, we wanted to elucidate the possible role of JNK in regulating K8/18 phosphorylation under physiological conditions, not involving unspecific stress. Therefore, we used stimulation of the FasR in HT-29 cells as a model, because in these cells FasR results in strong activation of JNK, without leading to apoptosis. By using this model, we observed that K8 is phosphorylated by JNK both in vitro and in vivo. K8 phosphorylation in vivo was observed after stress-activated JNK and following physiological JNK activation by stimulation of the FasR. The in vivo phosphorylation pattern of K8 follows the activation kinetics of JNK. Furthermore, JNK and K8/18 are associated with each other in vivo, as determined by co-immunoprecipitation studies. These results show that JNK participates in the regulation of K8/18 polymers. The association of JNK with K8/18 polymers may have a role in regulating JNK signaling and in adaptation to both environmental and physiological stresses.

**EXPERIMENTAL PROCEDURES**

**Materials**—PD98059 (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) were purchased from Calbiochem. Anisomycin was purchased from Calbiochem. Cycloheximide, myelin basic protein (MBP), and protein A-Sepharose were obtained from Sigma. Calcinucin A was from Alexis (Alexis Corp., Switzerland), and SB203580 was from Smith Kline & French Laboratories. Protein G-Sepharose, secondary horseradish peroxidase-conjugated anti-mouse antibody, Western blotting detection reagents, and [γ-32P]ATP were purchased from Amerham Biosciences. Fusion protein of glutathione S-transferase-5′-3′ of human c-Jun and c-jun-conjugated antibody was purified from Escherichia coli cells harboring the GST-c-Jun and GST-ATF-2 expression plasmid (kindly provided by Dr. James Woodgett, Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, Ontario, Canada). Polyclonal rabbit and goat antibodies to JNK or goat to p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibody generated against ERK2 (Transduction Laboratories, Lexington, KY) was used. Mouse antibody generated against human FasR immunoglobulin M antibody was from Kaniya Biomedical Science, Woodgett, Ontario Cancer Institute, Department of Medical Biophysics, Canada. For immunoblot analysis of phosphorylated substrates, the radioactive [γ-32P]ATP was used as a substrate and were boiled for 3 min to inactivate any endogenous kinase activity. The reaction was carried out at 37°C for 30 min in kinase reaction buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.5 mM EDTA, 1 mM PMSF, 1 mM sodium deoxycholate, 1 mM NaN3, 0.1% SDS, 1 mM EDTA, 1 mM EDTA, 20 mM NaF, 1 mM PMFS, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). For kinase immunoprecipitation, cell lysates were centrifuged at 3000 × g for 5 min, and the supernatant was incubated with antibodies against JNK, coupled to protein A-Sepharose, and against ERK2 or p38, coupled to protein G-Sepharose. Immunoprecipitates were then washed three times in RIPA buffer, three times in LiCl buffer (500 µM LiCl, 100 mM Tris, pH 7.6, 0.1% Triton X-100, 1 mM DTG), and three times in JNK/p38 kinase assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.5 mM EDTA) and ERK kinase assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 5 mM dithiothreitol, respectively). The kinase reaction was carried out by adding the immunoprecipitates 20 µl of glutathione S-transferase-5′-3′ of human c-Jun or K8 protein (150 g) or 3 µg of c-Jun GST protein (or 3 µg of GST-ATP or 3 µg of MBP as substrates). The reaction was carried out for 20 min at 37°C and stopped with 3× Laemmli sample buffer (30% glycerol, 3% SDS, 0.19 M Tris-HCl, pH 6.7, 0.015% bromophenol blue, 3% β-mercaptoethanol). The samples were resolved on a 12.5% SDS-PAGE. Quantification of phosphorylation of c-Jun, ATF, and MAPK was carried out with a PhosphoImager (Bio-Rad).

In Vitro Phosphorylation of K8/18—JNK was immunoprecipitated as described in the kinase activity assay from control and 9-h FasR-stimulated HT-29 cells. K8/18 protein preparations were used as a substrate and were boiled for 3 min to inactivate any endogenous kinase activity. The reaction was carried out at 37°C for 30 min in kinase reaction buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 25 µM ATP, 0.5 mM DTT) in the presence or absence of 2 µM of [γ-32P]ATP. This was followed by the addition of 3× Laemmli buffer to terminate the reaction. Phosphoproteins were then separated on 10% SDS-PAGE. Quantification of phosphorylation of c-Jun, ATF, and MAPK was carried out with a PhosphoImager (Bio-Rad).

**Immunoblotting and Immunoprecipitation Analysis**—For Western blotting, equal amounts of proteins were loaded on 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. After blocking overnight at 4°C with 1% BSA/1% Tween 20/PBS, membranes were incubated at 22°C for 1 h with the primary antibodies as follows: 1) JNK-antibody (Transduction Laboratories); 2) anti-MAPK (Santa Cruz Biotechnology); 3) anti-anti-human FasR immunoglobulin M antibody was from Kanine Biomedical Co. (Thousand Oaks, CA), and monoclonal anti-c-Jun was from Transduction Laboratories. Monoclonal rabbit antibodies specific for the phosphorylated and nonphosphorylated forms of ERK2/1 and p38 kinase were obtained from New England Biolabs Inc. (Beverly, MA). Secondary anti-rabbit horseradish peroxidase-conjugated antibody was purchased from Zymed Laboratories Inc. (Zymed Laboratories Inc., San Francisco, CA). Other monoclonal antibodies used included anti-nodulin (51), anti-pK8-Ser-73, and other anti-keratin and anti-phospho-keratin antibodies (3, 9, 43).

**Cell Culture**—Human HT-29 colon carcinoma cells and Jurkat T cells were obtained from American Type Cell Culture and were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium, respectively. The cell culture was supplemented with 10% heat-inactivated bovine fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine. Cells were maintained in a humidified incubator with 5% CO2 in air at 37°C. For Fas stimulation, cells were incubated with an agonistic anti-human FasR immunoglobulin M antibody (100 ng/ml) for the indicated times. For combination treatment, cells were additionally treated with (CHX). When indicated, cells were irradiated for 5 min with UV light or preincubated for 30 min with the irreversible general-caspase inhibitor Z-VD-FMK (20 µM), p38 kinase inhibitor SB203580 (10 µM), or with the MMKI kinase inhibitor PD98059 (50 µM).

**Keratin Preparations**—HT-29 cells (2 × 107/ml) were washed with PBS and harvested in ice-cold homogenization buffer (0.6 M KCl, 20 mM Hepes, pH 7, 1% Triton X-100, 1 mM MgCl2, 5 mM EDTA, 1 mM PMFS, 10 µg/ml leupeptin, 10 µg/ml antipain, 10 µg/ml pepstatin, 1 µg/ml aprotinin). After centrifugation at 3000 × g for 10 min at 4°C, the pellet was resuspended in disassembly buffer (8 mM urea, 10 mM Heps, 0.2% 2-mercaptoethanol, 1 mM EDTA, 1 mM PMFS, pH 7.4) and sonicated on ice. The digest was centrifuged again for 10 min at 4°C. The supernatant was dialyzed at 4°C for 2 h and against dialysis buffer (10 mM Heps, 0.2% 2-mercaptoethanol, 1 mM EDTA, 0.2 mM PMFS, pH 7.4). Reassembled IF proteins (keratins 8 and 18) were stored at −70°C.

**Kinase Activity Assay**—Cells (2 × 106/sample) were lysed with 400 µl of RIPA lysis buffer (PBS, 5 M NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM NaN3, 0.1% SDS, 1 mM EDTA, 1 mM EDTA, 20 mM NaF, 1 mM PMFS, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min with the irreversible general-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) for the indicated times. For combined treatment, cells were irradiated for 5 min with UV light or preincubated with 5% CO2 in air at 37°C for 30 min with the irreversible general-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) at 37°C. The digested peptides were washed with sterile water and dried with a speed vacuum. The digested samples were separated on microcrystalline-cellulose TLC plates (Merck) in two dimensions. The first dimension utilized electrophoresis and the second utilized chromatography, followed by autoradiography. For mixing experiments, equal counts of in vivo and in vitro
K8 Is Phosphorylated on Ser-73 Upon FasR Stimulation, and This Phosphorylation Occurs within the Soluble Keratin Pool—Keratins have been shown to be hyperphosphorylated under various conditions, including heat stress, virus infection, and stress-induced apoptosis. To investigate whether this is true for death receptor-mediated signaling, we stimulated the FasR and followed the time course of K8 phosphorylation in HT-29 cells, which are insensitive to FasR-mediated apoptosis but can be sensitized to undergo apoptosis by CHX (14). HT-29 cells were incubated with anti-FasR antibody alone or in combination with CHX at the indicated time points (Fig. 1A). A specific monoclonal antibody, LJ4, recognizing the phosphorylated Ser-73 on K8 was used, because this site is phosphorylated during heat shock and chemically induced apoptosis in HT-29 cells (9). K8 became hyperphosphorylated 3 h after FasR ligation, reaching a plateau of ~7-fold activation at 9 h, then remaining elevated up to 24 h after FasR stimulation. This was not due to increased synthesis of K8, as the protein levels remained constant (Fig. 1A, lower panel). Combined treatment of anti-FasR and CHX for 6 h resulted in similar K8 phosphorylation, indicating that K8 phosphorylation upon Fas cross-linking occurs regardless if cells are undergoing apoptosis or not and indicating that there is no additive effect by the fact that the apoptotic machinery is triggered. Similarly, we observed a strong K8 phosphorylation when we subjected HT-29 cells to unspecific stress by exposure to UV light (compare 1st and 2nd lanes in Fig. 1A).

Phosphorylation of K8 and 18 is often accompanied by an increase in keratin solubility (2, 26, 27). To test for this possibility, we prepared cell extracts from HT-29 cells and separated the pellet, containing insoluble keratins, and the supernatant, containing soluble keratins (see “Experimental Procedures”). As demonstrated in Fig. 1B, no increase of K8-Ser-73 phosphorylation after FasR stimulation was detected in the insoluble keratin pool, even when cells were stimulated with UV light. On the contrary, hyperphosphorylation of K8 on Ser-73 upon FasR stimulation was observed in the pool of soluble keratins (Fig. 1A). This was further confirmed by an additional centrifugation of the soluble keratin pool at 200,000 × g for 1 h. In this assay, all long and short keratin filaments will be pelleted, whereas the truly soluble subunits stay in the supernatant. As shown in Fig. 1D, hyperphosphorylation of K8 on Ser-73 in response to FasR stimulation occurred preferentially in the soluble pool (3rd and 4th lanes), whereas this phosphorylation remained nearly invariable in the insoluble fraction. These results clearly demonstrated that the depolymerized subunits are the target for phosphorylation, and therefore, we have employed the soluble keratin pool for further investigations.

Activation of JNK Induced by FasR Stimulation—JNK has been shown to be activated by FasR stimulation in many cell lines, both when cells are insensitive or sensitive to FasR-mediated apoptosis. (14, 28). Therefore, JNK was an obvious candidate kinase for the FasR-induced K8 phosphorylation. We followed the activation kinetics of JNK after stimulation of the FasR in HT-29 cells. Parallel samples were taken from treatments described above (Fig. 2A, upper panel). Consistent with the results on K8 phosphorylation, both combined treatments of anti-FasR and CHX and UV light were able to activate JNK. The amount of JNK was confirmed by immunoblotting by using anti-JNK antibody that recognizes both the 46- and 54-kDa isoforms. A histogram of relative K8 phosphorylation and JNK activation (Fig. 2B) showed closely corresponding kinetics and levels of induction (Fig. 1C). Thus, it seemed plausible that JNK would be regulating the phosphorylation of K8 in response to FasR stimulation.

Phosphorylation of K8 on Ser-73 by JNK in Vitro—If the hypothesis of JNK being a regulator of K8 is correct, JNK should also be able to phosphorylate K8 in vitro. We verified this by immunoprecipitating JNK from control cells and from cells 9 h after FasR stimulation. K8/18 isolated from HT-29 cells was used as a substrate for immunoprecipitated JNK. The autoradiograph in Fig. 3A (upper panel) shows an elevated phosphorylation of both K8 and K18 by Fas-activated JNK; however, the phosphorylation is significantly more pronounced on K8. Next, we examined whether this phosphorylation included K8 Ser-73. For that purpose, the preparation of isolated K8/18 was phosphorylated by immunoprecipitated JNK in the absence of [γ-32P]ATP and subjected to immunoblot analysis using the LJ4 antibody (Fig. 3A, middle panel). We could
clearly see that the in vitro phosphorylation of K8 by JNK occurred on Ser-73, and this phosphorylation was markedly elevated upon FasR stimulation. Equal loading of K8/18 was confirmed by reblotting the same nitrocellulose membrane with anti-K8/18 antibodies (Fig. 3A, lower panel).

To further substantiate that K8 is an in vivo JNK target, tryptic phosphopeptide maps of K8 immunoprecipitated from 32P in vivo labeled HT-29 cells and of K8 phosphorylated by JNK in vitro were compared. Seven peptides (peptides 2–8) could be found in the maps of control cells and in those obtained from anti-FasR antibody stimulated cells (Fig. 3B), with enhanced activities primarily on peptides 3 and 5 within the 7 cycles was considered as phospho-Ser-73-containing peptides. Only peptides 3 and 10 met these criteria (Table I). Peptide 2, which was not inducibly phosphorylated and phosphorylation of K8 by JNK. To identify which of these peptide(s) corresponded to Ser-73, manual Edman degradation was performed. Due to partial digestion, several peptides can potentially correspond to Ser-73. Ser-73 is located at position of 27 on its specific tryptic peptide (not shown), which makes it inaccessible for cleavage because Edman degradation is inefficient after 10 cycles. In the present study, 7 cycles of manual Edman degradation were done for each peptide. The peptides showing inducible phosphorylation, both in vivo and by JNK in vitro, and no phospho-label release within the 7 cycles was considered as phospho-Ser-73-containing peptides. Only peptides 3 and 10 met these criteria (Table I). Peptide 2, which was not inducibly phosphorylated and showed very little phosphorylation by JNK in vitro, was considered to be Ser-431. This assignment was based on its sequence context (i.e., inaccessible to manual Edman degradation, not shown) and by the use of anti-phospho-Ser-431 K8 antibody (3), which showed that the phosphorylation of this site is not elevated by increased JNK activity in vivo or by JNK in vitro (data not shown). Peptide 6, a major inducible site in vivo, corresponds to Ser-23 of the tryptic peptide SYTSGPGSR. Hence, our peptide mapping data support the assignment of K8 Ser-73 as a major in vivo phosphorylation site for JNK upon FasR stimulation.

**Fig. 2.** JNK is concomitantly activated upon FasR stimulation. HT-29 cells were treated as stated in Fig. 1. A, JNK activation was measured by an immunocomplex kinase assay with c-Jun as a substrate (upper panel). Confirmation of an equal amount of the JNK kinase used for immunoprecipitation was determined by immunoblot analysis with an anti-JNK antibody (lower panel). B, comparison of the relative JNK kinase activity and K8-Ser-75 phosphorylation at the same time course after FasR stimulation. Bars represents the relative JNK activity and pK8-Ser-73 levels. c-Jun-associated 32P labeling was quantified by PhosphorImager analysis, and quantification of K8 phosphorylation was done by computer-assisted densitometry (MCID) using the Molecular Analyst Computer Program. The experiments were repeated three times with essentially identical results. Lane C, control.

**Fig. 3.** K8 is in vivo phosphorylated by JNK. An isolated K8/18 protein preparation from HT-29 cells was used as a substrate and phosphorylated in the presence or absence of [γ-32P]ATP by immunoprecipitated JNK from FasR-stimulated (9 h with anti-FasR antibody) and unstimulated HT-29 cells (A). Phosphoproteins were separated on 10% SDS-PAGE, followed by autoradiography or immunoblotting analysis. A, upper panel shows an autoradiograph of the phosphorylated K8 in the presence of [γ-32P]ATP. Phosphorylation of K8 in the absence of [γ-32P]ATP was also measured using a Western blot using LJ4 antibody (middle panel). Equal protein loading was confirmed by reblotting the same filter (nitrocellulose membrane) with anti-K8/18 antibodies (lower panel). B, phosphopeptide maps of K8 phosphorylated in vivo and in vitro. K8 was extracted from control [32P]orthophosphate-labeled HT-29 cells, or similarly labeled cells treated with anti-FasR antibody or in vitro phosphorylated by immunoprecipitated UV-activated JNK (see JNK assay). Samples were digested with trypsin and resolved by electrophoresis in pH 8.9 buffer in the first dimension (horizontal direction) and chromatography in the second dimension (vertical direction). The origin (ori) is indicated. Numbers indicate the corresponding peptides in the in vivo and in vitro maps. For mixing experiments, equal counts of in vivo and in vitro material were mixed and subjected for separation. Lane C, control.
panel confirmed equal protein loading.

When ERK activity was measured, no major increase was observed, as shown in the kinase assay with myelin basic protein (MBP) as a substrate (Fig. 4B, upper panel). The expression level of ERK protein appeared also to be unchanged as shown by immunoblotting (Fig. 4B, lower panel). The constitutive ERK activation in HT-29 cells is high because the type 2A phosphatase inhibitor cl-A failed to enhance the activity of ERK in these cells. To ensure that this observation was not due to a nonfunctional assay, we employed Jurkat T-cells, and we showed that cl-A activates ERK in this cell line (28) (Fig. 4C). Taken together, these results suggest that JNK is preferentially activated upon FasR stimulation in HT-29 cells and could be a potential kinase responsible for the K8 phosphorylation on Ser-73.

In vitro experiments were performed using ERK and p38 kinase immunoprecipitates that were obtained from control and Fas-stimulated cells. Immunoblot analysis with LJ4 antibody revealed that immunoprecipitated ERK or p38 kinase was able to phosphorylate K8 on Ser-73 in vitro (Fig. 4, D and E). However, this phosphorylation was not elevated in response to FasR activation. The lack of a role for ERK and p38 kinase in K8-Ser-73 phosphorylation in FasR-mediated signaling was further supported by additional experiments using specific inhibitors of both kinases. HT-29 cells were pretreated with PD98059, a specific inhibitor of the upstream regulator of ERK (20 nM) or serum starvation followed by 9 h of incubation with anti-FasR antibody. Phosphorylation of K8 on Ser-73 was analyzed by immunoblotting. As shown in Fig. 4, F and G, neither PD98059, nor SB203580 could inhibit K8-Ser-73 hyperphosphorylation after FasR stimulation. We conclude that the observed hyperphosphorylation of K8 on Ser-73 seems to be associated directly with JNK activation and occurs independently of ERK and p38 kinase.

Depending on the cell type, JNK activation after FasR stimulation can either be caspase-dependent (33) or -independent (15). We investigated whether JNK activation and JNK-mediated K8-Ser-73 phosphorylation upon FasR stimulation is caspase-dependent. HT-29 cells were preincubated with the irreversible general caspase inhibitor Z-VAD-FMK (34) at a concentration of 20 μM for 30 min, followed by ligandation of anti-FasR antibody for 9 h. The kinase assay did not reveal any inhibition of JNK following Z-VAD-FMK treatment (Fig. 5A). Likewise, no inhibition of JNK-induced K8-Ser-73 hyperphosphorylation was observed (Fig. 5B). Hence, FasR-mediated JNK activation and K8-Ser-73 hyperphosphorylation are caspase-independent in HT-29 cells.

In Vivo Interaction of JNK and Keratin 8 in HT-29 Cells—As

### Table I

**Manual Edman degradation of tryptic 32P-labeled K8 peptides**

Tryptic peptides were extracted from the TLC plates used for peptide mapping (see Fig. 3B), immobilized on membrane discs, and subjected to seven Edman degradation cycles. The table shows the cycle number at which the peak radioactivity was obtained for each respective peptide and whether there was radioactivity remaining on the disc after completion of seven cycles (D).

| Peptide no. | Edman degradation, label released on cycle number or remained on D (disc) | Total no. cycles |
|-------------|-------------------------------------------------|------------------|
| 2           | D                                               | 7                |
| 3           | D                                               | 7                |
| 4           | No data                                         | 7                |
| 5           | 2,3 and D                                       | 7                |
| 6           | 1 and D                                         | 7                |
| 7           | 2,3 and D                                       | 7                |
| 8           | 3 and D                                         | 7                |
| 9           | 3 and D                                         | 7                |
| 10          | D                                               | 7                |

**A. p38 kinase assay**

**B. ERK kinase assay**

**C.**

**D. In vitro phosphorylation of K8 by p38**

**E. In vitro phosphorylation of K8 by ERK**

**F.**

**G.**

**HT-29**

**Jurkat**

**FIG. 4.** p38 kinase and ERK are not involved in the phosphorylation of K8 in FasR-mediated signaling. HT-29 cells were treated as outlined in Fig. 1. A, activation of p38 kinase was measured by immunocomplex kinase assay with ATF-2 as a substrate (upper panel). The amount of nonphosphorylated p38 was determined by immunoblots with an anti-p38 antibody (lower panel). B, activation of ERK was measured by immunocomplex kinase assay with MBP as a substrate (upper panel). The loading of ERK2 was determined by immunoblots with an anti-ERK2 antibody (lower panel). C, for a positive ERK kinase assay, Jurkat cells were treated with cl-A (20 μM) for 30 min. In vitro phosphorylation of K8 by p38 kinase (D) and ERK (E), respectively, was determined as described in the legend of Fig. 2. Upper panels are Western blots by LJ4 antibody, and lower panels show equal protein loading. F, HT-29 cells were incubated with anti-FasR antibody for 9 h in the presence or absence of p38 kinase inhibitor SB203580 (SB) (20 μM) or serum starvation followed by 3 h of treatment with the Raf-MKK1-MAPK inhibitor PD98059 (PD) (50 μM) (G). Whole cell lysates from control and treated cells were separated on 10% SDS-PAGE and immunoblotted with LJ4 antibodies. Lane C, control.

JNK usually shows a high affinity for its substrates, we wanted to clarify whether this would apply for K8 as well. JNK was immunoprecipitated from control and Fas-treated HT-29 cells and then immunoblotted using anti-K8 antibody. As shown in Fig. 6A (upper panel), K8 was co-immunoprecipitated with JNK. The interaction was specific as no JNK was precipitated with control or FasR-stimulated cells. Immunoblot analysis with LJ4 antibody confirmed equal protein loading.

**FIG. 5.** JNK-mediated K8 phosphorylation on Ser-73 is caspase-independent. A, Western blot analysis with anti-FasR antibody. Phosphorylation of K8 on Ser-73 was detected among the co-immunoprecipitated K8 (Fig. 6A, middle panel), and the amount of co-immunoprecipitated pK8-Ser73 increased following FasR stimulation, as well as UV irradiation in HT-29 cells. The lower panel of Fig. 6A shows the immunoprecipitated JNK. This binding was not mediated by the heat shock protein 70, which associates with K8 (35), because heat shock protein 70 was not co-immunoprecipitated with JNK (data not shown). These results indicated that JNK not only phosphorylated K8 but also...
FIG. 5. Increase of JNK activity and hyperphosphorylation of the soluble pool of K8 on Ser-73 upon FasR stimulation is caspase-independent. HT-29 cells were pretreated with the irreversible pan-caspase inhibitor, zVAD-FMK (20 μM) for 30 min followed by 9 h of incubation with anti-FasR antibody. A, JNK activity was measured by immunocomplex kinase assay with c-Jun as a substrate. B, Western blot of phospho-K8 in RIPA buffer cell lysates by using LJ4 antibody. Lane C, control.

FIG. 6. JNK is associated with K8 in vivo. A, immunoprecipitates were prepared from HT-29 cells stimulated (9 h with FasR antibody) and unstimulated or were exposed to UV light for 5 min. Antibodies used were control rabbit IgG (1st lane) and anti-JNK antibody (2nd to 4th lanes). This was followed by immunoblotting with the LJ4 antibody (middle panel) as well as an anti-K8 monoclonal antibody (upper panel). The amounts of JNK pulled down were controlled by blotting with a goat anti-JNK antibody (lower panel). B, K8 and LJ4 were immunoprecipitated (IP) from HT-29 cells, and the immunoprecipitation was repeated twice. The supernatant (sup) and pellet (pel) obtained each time were resolved on SDS-PAGE and blotted with anti-JNK antibody. Monoclonal anti-nodularin was used as an antibody control. C, HT-29 cells were untreated or treated with anti-FasR antibody for the indicated time points, and cell lysates were immunoblotted with antibodies to the endogenous c-Jun. The retarded migration seen in the sample with anisomycin-treated (Ani) (5 μg/ml) for 20 min (Fig. 6C). However, the possibility that targeting of JNK to K8 could regulate the degree of c-Jun activation requires a more detailed analysis beyond the scope of the present study.

**DISCUSSION**

Our studies show that K8 Ser-73 is phosphorylated in response to FasR stimulation in HT-29 cells. This phosphorylation is likely to be regulated by JNK, as demonstrated by both in vitro and in vivo studies. In the context we studied, ERK and p38 kinase did not appear to play a role, as reflected by the failure to detect increases in their activities in response to FasR stimulation. Furthermore, K8/18 associate with the 54-kDa JNK and could thereby participate in regulating its functions.

**K8 as a Substrate for JNK**—Although JNK has been considered to be mainly a transcription factor kinase, some cytoplasmic cytoskeletal substrates have also been reported. A strong correlation between hyperphosphorylation of a neuronal IF protein, neurofilament high molecular weight subunit (NF-H), and activation of JNK3 has been demonstrated, thereby suggesting that the NF-H C-terminal domain was phosphorylated by JNK3 (25). Also, the neuronal microtubule-associated protein, tau, is phosphorylated in vitro by JNK at sites that are hyperphosphorylated in tau from patients with Alzheimer’s disease, as compared with fetal or adult tau (36). More recently, another possible JNK-specific tau phosphorylation site has been postulated (37). Despite these implications, there are no data to firmly establish JNK-mediated phosphorylation of a cytoskeletal target in vivo. Using the specific LJ4 antibody and specific phosphopeptide mapping, we have shown that upon FasR stimulation K8 is hyperphosphorylated on Ser-73 in a JNK-dependent manner.

Previous results (9, 38, 39) have shown that the kinase responsible for the phosphorylation of Ser-73 could involve a kinase from the MAPK family, due to the Ser-Pro motif of K8-Ser-73 phosphoacceptor site, which could serve as an in vitro target for proline-directed MAPK/ERK. When comparing the amino acid sequence surrounding the Ser-63 and Ser-73 phosphoacceptor sites in the c-Jun transcription factor with that surrounding Ser-73 in K8 (Table II), essential similarities were observed. Hence, the Ser-73 phosphopeptide on K8 is a likely phosphorylation site for JNK. However, despite phosphoacceptor sequence similarities, specific and effective phosphorylation by JNK requires a specific docking site as shown for the JNK substrates, c-Jun and ATF-2 (39–41). In our study, we demonstrated that K8 and JNK co-immunoprecipitated with high affinity. In searching for possible docking sites in K8,
we compared the docking site sequences from a number of JNK substrates with the sequences of K8 and K18, and we found that there are two short sequences in K8 and one in K18 that resemble the docking sequences of these JNK substrates (Table III). Thus, K8 could either bind to JNK directly by itself or indirectly via polymerization with K18 that interacts with JNK, as described for JunD (38). However, this interaction does not seem to go through heat shock protein 70, which has been shown in complex with K8 (35). Furthermore, K8 tends to associate more efficiently with the 54-kDa isoform of JNK, indicating substrate specificities of JNK isoforms for K8. Recently, a cytosplactic scaffold protein JIP1, which binds selectively to JNK and to the upstream activating kinases, but not to other related MAPK including p38 and ERK, has been discovered (23). Overexpression of JIP1 causes cytosplactic retention of JNK and suppresses the effects of JNK on apoptosis (42). In our study, we did not observe any significant elevation of endogenous c-Jun phosphorylation upon FasR stimulation in HT-29 cells, although JNK was highly activated following the same treatment. This raises the possibility that in some tumor cell lines, such as HT-29 cells, K8/18 may act as a JNK sequestering complex, thereby weakening JNK from performing some of its cytosolic or nuclear tasks. However, the possibility that the observed association of JNK with K8 would affect JNK targeting needs to be examined in closer detail before any solid conclusions can be drawn.

In agreement with previous reports (8, 9), we have shown that K8-Ser-73 is a site that can be phosphorylated by ERK and p38 kinases in vitro. However, phosphorylation of this K8 site by ERK and p38 kinase was not enhanced, and the activities of these two kinases were not elevated in response to FasR stimulation. Thus, the observed K8 phosphorylation does not seem to be ERK- or p38 kinase-mediated but is JNK-dependent. However, K8 Ser-73 can also be a selective in vivo p38 kinase substrate under conditions that activate this kinase (55).

### Table III

| K8       | K18       | c-Jun    | ATF-1   | JIP-1    |
|----------|-----------|----------|---------|----------|
| RQLETLGGEKLLKLEAEGLN | MNVKLADIEATYRLKL | LKRVIDDNTIRLQLET | NPRLIKGSMTLTNLADPVSGLK | LAVHKKHHMTTLKGPAPA |
| 143      | 182       | 236      | 256     | 296      |

The Possible Biological Functions of K8-Ser-73 Phosphorylation Mediated by JNK—The significance of keratin phosphorylation has been extensively studied (1, 43). One common feature of keratin phosphorylation is the increase in their solubility (43). In the present study, a basal level of phosphorylation on K8-Ser-73 in control and stimulated HT-29 cells was discerned in the nonsoluble keratin pool, whereas the increase in Ser-73 hyperphosphorylation was observed almost exclusively in the pool of disassembled keratin IFs. This enhanced phosphorylation and solubility is likely to be of importance in regulating keratin turnover, assembly, and dynamics. The execution of apoptosis requires cleavage of a number of proteins, such as keratins. In a human endometrial adenocarcinoma cell line SNG-M, K18, but not K8, was degraded by caspases during apoptosis (44). Similarly, in HT-29 cells, K8 was protected from cleavage when phosphorylated on Ser-73, whereas K18 was degraded regardless of its phosphorylation state (45). Furthermore, our results showed that the K8 hyperphosphorylation and JNK activation upon FasR stimulation were both apoptosis- and caspase-independent. Recently, keratin-dependent, JNK-mediated epithelial resistance to TNF-mediated apoptosis has been reported. K8 and K18 bind to the cytoplasmic domains of TNF receptor 2 and modulate the TNF-dependent activation of JNK and the NFκB transcription factor. Cells with the truncated form of K18 and disrupted K8/18 filaments responded to TNF by increasing the level and the duration of JNK activity. Thus, epithelial cells with decreasing amounts of K8/18 display a higher cellular sensitivity to killing by TNF (46). Taken together, it is possible that upon FasR stimulation, JNK-mediated caspase-independent hyperphosphorylation of K8 on Ser-73 would be involved in protecting the keratin network from cleavage by caspases in the FasR-resistant HT-29 cell line.

One important feature of JNK in regulation of various transcription factors, such as c-Jun, JunB, ATF-2, or tumor suppressor p53, is to target them for ubiquitination, and the interaction between JNK and these proteins appears to be essential for the targeting. Disruption of these bindings results in prolonged half-lives of these transcription factors (47, 48). Under stress conditions, phosphorylation of these proteins is enhanced by activated JNK, and the ubiquitination is thereby repressed, so that these transcription factors can function to cope with stress (49). A recent report has also illustrated that phosphorylation of K8 on Ser-431/-73 and of K18 on Ser-52 protects them from ubiquitination (50). Therefore, JNK may be involved in regulating K8 ubiquitination by interacting with the keratin filaments and phosphorylating K8, and this regulation may be important for cells to withstand various stresses.

In conclusion, this study illustrates the existence of a novel cytoskeletal substrate for JNK in epithelial cells. The soluble pool of K8 is hyperphosphorylated on Ser-73 by JNK upon FasR stimulation in a caspase- and apoptosis-independent manner in HT-29 cells. Although the role for this phosphorylation is still not clear, the JNK/K8 interaction could be important in both controlling K8/18 dynamics and turnover as well as in the control of JNK signaling.

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