α-Thrombin Rapidly Induces Tyrosine Phosphorylation of a Novel, 74–78-kDa Stress Response Protein(s) in Lung Fibroblast Cells*

Received for publication, August 6, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, September 13, 2004, DOI 10.1074/jbc.M409043200

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We demonstrated previously that exposure of CCL39 lung fibroblasts to α-thrombin rapidly inhibits interleukin 6-induced tyrosine phosphorylation of signal transducers and activators of transcription 3 (Stat3). While studying the cross-talk between α-thrombin and interleukin 6, we observed that the phospho-specific (tyrosine) anti-Stat3 antibody specifically cross-reacted with a 74–78-kDa protein(s) in α-thrombin-treated cells. In this study, we demonstrate that in α-thrombin-treated CCL39 cells, the 74–78-kDa protein(s) rapidly undergoes tyrosine phosphorylation. The phosphorylation by α-thrombin was detected as early as 5 min and reached a maximum at 15 min; however, low levels were present at 2 h. α-Thrombin receptor agonist peptide (8FLLRN) induced its tyrosine phosphorylation, suggesting that α-thrombin mediates the effects via protease-activated receptor type 1. Anti-Stat3 antibodies specific to different regions of Stat3 failed to recognize the 74–78-kDa protein(s), suggesting that it is unrelated to Stat3. Cell fractionation experiments showed that it is localized to the cytoplasm. Mass spectrometric analysis of the immunoprecipitated protein showed that the 74–78-kDa protein(s) is related to glucose-regulated protein 75 (GRP-75), a member of the heat shock/stress-response protein family. Consistent with these data, we observed tyrosine phosphorylation of GRP-75 in α-thrombin-treated cells. Exposure of cells to pterovadate, a stress-inducing agent, stimulated its tyrosine phosphorylation; however, cytokines and growth factors were ineffective. This is the first report of tyrosine phosphorylation of GRP-75-related stress protein(s) by α-thrombin and suggests that this pathway may contribute to the ability of α-thrombin to prevent apoptosis in cells exposed to stress or in the injured tissue.

α-Thrombin generated at sites of vascular injury is a serine protease central to blood coagulation. However, it also induces a variety of cellular responses in cardiovascular and pulmonary cell types (1). It causes platelet activation (2–4), is chemotactic to monocytes, and is mitogenic for lymphocytes, fibroblasts, and vascular smooth muscle cells (5–7). It acts on vascular endothelium to stimulate the production of prostacyclin, platelet-activating factor, and platelet-derived growth factor (PDGF) (2). It also induces the production of interleukin-1 (IL-1) and interleukin-6 (IL-6) from macrophages, fibroblasts, and endothelial cells. Thus, α-thrombin is an important mediator of not only hemostatic but also inflammatory and proliferative/reparative responses to tissue injury. A family of G-protein-coupled protease-activated receptors (PARs) mediates cellular actions of α-thrombin (8–11). Although α-thrombin can activate three of the four PAR family members, PAR-1 represents the primary thrombin-responsive receptor in human cells. α-Thrombin binding to its receptor activates heterotrimeric G-proteins and stimulates many signals including activation of phospholipase C with generation of inositol triphosphate and an increase in intracellular calcium (2). Other signaling intermediates activated by α-thrombin include the following: tyrosine kinases such as Src kinases, focal adhesion kinase, and JAK kinases and serine-threonine kinases such as protein kinase C, p42/44 kinases, and p38 kinases (7, 12–17). It also activates protein-tyrosine phosphatases 1D, 18, 19. These intermediates either individually or collectively modulate the activity of different substrates, including transcription factors that cause distinct cellular responses.

In a previous study (20), we demonstrated that exposure of cells to α-thrombin inhibits IL-6-induced Stat3 tyrosine phosphorylation in CCL39 and MRC-5 lung fibroblasts. The α-thrombin-mediated inhibition required activation of mitogen-activated protein kinase kinase 1 (20). This inhibitory pathway was also observed between angiotensin II and IL-6 (21, 22) and suggests that in few cell types, G-protein-coupled receptors by activating mitogen-activated protein kinase pathway have the ability to modulate IL-6-induced signaling. Stat3 protein is a member of the STAT family of transcription factors, and its activation by tyrosine phosphorylation is a critical step in IL-6-induced signaling (23). While studying the inhibitory effect of α-thrombin on IL-6 signaling, by serendipity, we observed that, in addition to tyrosine-phosphorylated Stat3, the phospho-specific Stat3 antibody cross-reacted with a 74–78-kDa protein(s) in α-thrombin-treated cells. Enhanced cross-reactivity of the phospho-specific Stat3 antibody with the 74–78-kDa protein(s) in multiple experiments suggested that α-thrombin may regulate its phosphorylation. Therefore, we initiated studies to establish the identity of the 74–78-kDa protein and its relation to Stat3. We were also interested to determine whether it has a role in α-thrombin-mediated inhibition of IL-6-induced Stat3 tyrosine phosphorylation.

In this study, we demonstrate that the 74–78-kDa protein(s) rapidly undergoes tyrosine phosphorylation in response to α-thrombin. It was localized to cytoplasm in both unstimulated...
and α-thrombin-stimulated cells. The interaction of the phospho-specific antibody with the 74–78-kDa protein(s) was specific; however, it was unrelated to the Stat3 transcription factor. Mass spectrometric analysis showed that the 74–78-kDa protein is related to GRP-75, a member of heat shock protein (HSP)/stress-response protein family. Pervanadate, a stress-inducing agent, also induced its tyrosine phosphorylation. Stat3 failed to co-precipitate with the 74–78-kDa protein(s), suggesting this protein(s) is unlikely to be involved in the inhibition of IL-6-induced Stat3 signaling by α-thrombin. Our results demonstrate that α-thrombin regulates tyrosine phosphorylation of GRP-75-related stress-response protein(s) in lung fibroblasts. This novel pathway may contribute to α-thrombin-mediated proliferative/reparative responses observed following tissue injury.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Invitrogen. IL-6, PDGF, transforming growth factor-β (TGF-β), and basic fibroblast growth factor (FGF) were obtained from R & D Systems; nitrocellulose membranes from Amersham Biosciences; phospho-specific anti-Stat3 antibody was from BIOSOURCE; anti-Stat3 antibody and anti-E2F-1 antibody were from Santa Cruz Biotechnology; anti-phosphotyrosine antibody was from Upstate Biotechnology, Inc.; anti-GRP-75 antibody was from Oxford Biomedical Research; goat anti-rabbit IgG and rabbit anti-mouse IgG were from Bio-Rad; all other chemicals were either from Sigma or Fisher.

Cell Culture—CCL-39 cells were obtained from American Type Culture Collection and grown in McCoy’s 5A medium containing 10% fetal bovine serum. Cells were grown for 12–24 h and serum-starved for 12 h in McCoy’s medium before the addition of α-thrombin or cytokines.

Immunoprecipitation and Western Blots—Cells were treated with various agents for the indicated times and washed with phosphate-buffered saline. Cells were scraped in lysis buffer (10 mM Tris-Cl, pH 7.4, 1% Triton X-100, 15% glycerol, 1% N,N-dimethylformamide, 1 mM sodium orthovanadate), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of proteins were immunoprecipitated with anti-phosphotyrosine antibody/anti-GRP-75 antibody and protein A/G-agarose. Immunocomplexes were harvested by centrifugation and washed three times with immunoprecipitation buffer (lysis buffer), and proteins were resolved by 8% polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with their respective primary antibodies. Immunoreactive bands were visualized using a chemiluminescence Western blotting system according to the manufacturer’s instructions (Amersham Biosciences).

Cell Fractionation—All cell fractionations were carried out at 4 °C. Cells were harvested by scraping into ice-cold phosphate-buffered saline, washed twice with cold phosphate-buffered saline, resuspended in saline, resuspended in 100 μl of hypertonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 3 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) per cell pellet derived from 200-mm cultures. Using a loose fitting Dounce homogenizer, cells were gently broken to fracture into cytoplasmic and nuclear fractions as described earlier (24). Nuclei were removed from the cell homogenate by low speed centrifugation (1000 rpm for 4 min) in an Eppendorf centrifuge. The crude post-nuclear cytoplasmic fraction was further subjected to 15,000 × g in an Eppendorf tube for 15 min. The pellet represented the membrane fraction (mitochondria, endoplasmic reticulum, and plasma membrane). The supernatant represented the cytoplasmic fraction.

Mass Spectrometry Analysis—The immunoprecipitated protein was separated on an 8% SDS-polyacrylamide gel, and stained with Coomassie Blue. The stained gel bands were rinsed in water, cut into 1-mm pieces, dehydrated with 0.2 N NH4HCO3, 50% acetonitrile for 30 min, and dried in a Speed-Vac. The gel pieces were then rehydrated in 0.1 M NH4HCO3 containing 0.5–1 μg of modified trypsin (Promega) and digested for 20 h at 37 °C. The supernatant was removed to a clean microcentrifuge tube, and the gel fragments were extracted with aqueous 50% methanol, 1% formic acid for 30 min and combined with the initial extract. This was evaporated to ~20 μl and desalted on a C18 ZipTip (Millipore) as recommended by the vendor. Peptides were eluted from the ZipTip with a 2–5-μl aqueous solution of 50% methanol and 1% formic acid. One μl was mixed with matrix (α-cyano-4-hydroxycinnamic acid) and spotted on a MALDI target plate for analysis by Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer. Peptide masses detected were sent to PROWL (Rockefeller University) or Protein Prospector (University of California, San Francisco) for protein data base searches for possible matches. The search was done for all species (in the NCBI data base) for a molecular mass range of 5–250 kDa (wide open search).

Sequence Analysis—Sequence comparison was performed using the BLAST search program, and the search was done with NCBI nr data base.

RESULTS

Phospho-specific (Tyrosine) Anti-Stat3 Antibody Cross-reacts with the 92-kDa Stat3 Protein in IL-6-treated Cells; However, It Detects a 74–78-kDa Protein(s) in α-Thrombin-treated Cells—In a previous study, we demonstrated that pretreatment of CCL39 and MRC-5 lung fibroblasts with α-thrombin inhibited IL-6-induced Stat3 tyrosine phosphorylation (20). We initiated the present study to further understand the molecular mechanism of this inhibition. Since the commercially obtained phospho-specific anti-Stat3 antibody (specific for tyrosine-phosphorylated Stat3) represented a new batch, we first sought to reproduce the inhibitory cross-talk between α-thrombin and IL-6 by using this antibody. This antibody was raised against a phosphopeptide of Stat3 (SAPPA*LYTKTF, where Y* indicates phosphotyrosine) corresponding to amino acids 701–710. Total cell extracts were prepared from untreated cells or cells treated with α-thrombin alone or IL-6 alone or with α-thrombin and IL-6. Equal amounts of proteins were immunoblotted with the phospho-specific anti-Stat3 antibody. Fig. 1A demonstrates that this antibody cross-reacted with the 92-kDa Stat3 protein only in IL-6-stimulated cells (lane 3), and a corresponding protein was absent in unstimulated control cells (lane 1). This result shows that the phospho-specific anti-Stat3 antibody recognizes tyrosine-phosphorylated Stat3 in IL-6 treated cells. Consistent with our earlier findings (20), α-thrombin treatment alone failed to induce Stat3 tyrosine phosphorylation (Fig. 1A, lane 2), and pretreatment of cells with α-thrombin completely inhibited IL-6-induced Stat3 tyrosine phosphorylation (Fig. 1A, lane 4).

However, to our surprise, we observed in Fig. 1A that the phospho-specific anti-Stat3 antibody also cross-reacted with a 74–78-kDa protein(s), particularly in α-thrombin (lane 2), and α-thrombin + IL-6 (lane 4)-stimulated cells. This protein(s) was weakly detected in control (Fig. 1A, lane 1) and IL-6 (Fig. 1A, lane 3)-treated cells. The increased intensity of the 74–78-kDa protein(s) in α-thrombin (Fig. 1A, lane 2)-treated compared with unstimulated cells (lane 1) suggested that α-thrombin regulates this protein(s) via phosphorylation. To determine whether the 74–78-kDa protein(s) is related to Stat3, we stripped the blot in Fig. 1A and reprobed with an anti-Stat3 antibody specific to the NH2-terminal region of Stat3. This antibody is expected to detect total Stat3 protein, including both unphosphorylated and phosphorylated forms. Fig. 1B demonstrates that the NH2-terminal specific anti-Stat3 antibody cross-reacted with a 92-kDa protein in all lanes, which co-migrated with the tyrosine-phosphorylated 92-kDa protein observed in Fig. 1A, lane 3. This shows that all lanes in Fig. 1A contained similar amounts of Stat3 protein. However, the NH2-terminal specific anti-Stat3 antibody failed to detect the 74–78-kDa protein(s) in all lanes (Fig. 1B). In additional experiments, we reprobed the blot with anti-Stat3 antibodies raised against the COOH terminus and an internal region of the Stat3 protein. Once again, these anti-Stat3 antibodies detected the 92-kDa Stat3 protein but failed to detect the 74–78-kDa protein(s) (data not shown). These results demonstrate that the 74–78-kDa proteins recognized by the phospho-specific anti-Stat3 antibody in Fig. 1A are distinct and unrelated to Stat3. Collectively, these findings suggest that the 74–78-kDa protein(s) contains an antigenic epitope that is selectively recognized by the phospho-specific anti-Stat3 antibody.
To determine the specificity of interaction of the phosphospecific anti-Stat3 antibody with the 74–78-kDa protein(s), we tested if preincubation of the antibody with phosphorylated Stat3 peptide (immunogen) would abolish its ability to cross-react with 74–78-kDa protein(s) in immunoblots. As a control, we also preincubated the phospho-specific anti-Stat3 antibody with the corresponding unphosphorylated Stat3 peptide. Fig. 1C demonstrates that preincubation of phosphospecific anti-Stat3 antibody with the Stat3 phosphopeptide abrogates its ability to cross-react with the 74–78-kDa protein(s). However, when phosphospecific anti-Stat3 antibody was incubated with unphosphorylated Stat3 peptide, it still retained the cross-reactivity with the 74–78-kDa protein(s) (Fig. 1D). These results show that the interaction between phosphospecific anti-Stat3 antibody and the 74–78-kDa protein(s) is specific and that the antibody recognition motif in the 74–78-kDa protein(s) may contain a phosphotyrosine residue similar to Stat3.

The 74–78-kDa Protein(s) Does Not Co-precipitate with Stat3—We considered the possibility that the 74–78-kDa protein(s) may interact/associate with Stat3 to cause inhibition of IL-6-induced Stat3 tyrosine phosphorylation by α-thrombin in CCL39 cells (20). To address this possibility, we tested if Stat3 would co-precipitate with the 74–78-kDa protein(s). Lysates were prepared from unstimulated cells or cells stimulated with α-thrombin, IL-6, or α-thrombin + IL-6. Proteins were immunoprecipitated with phospho-specific anti-Stat3 antibody and immunoblotted with anti-Stat3 antibody. Fig. 1E demonstrates that Stat3 does not co-precipitate with the 74–78-kDa protein(s). As a control for the immunoprecipitations, we reprobed the blot in Fig. 1E with phospho-specific anti-Stat3 antibody. Fig. 1F shows that the phospho-specific anti-Stat3 antibody immunoprecipitates the 74–78-kDa protein(s) particularly in α-thrombin (lane 2) and α-thrombin + IL-6 treated cells (lane 4). Phosphospecific anti-Stat3 antibody immunoblots of immunoprecipitated Stat3 also failed to show co-precipitation of Stat3 with the 74–78-kDa protein(s) (data not shown). Lack of a direct interaction between these two proteins suggest that the 74–78-kDa protein(s) may not be involved in α-thrombin-mediated inhibition of IL-6-induced Stat3 tyrosine phosphorylation.

It is also important to note that the number of protein bands in the 74–78-kDa range detected by the phospho-specific anti-Stat3 antibody slightly varied between experiments. For example, in Fig. 1A, the phospho-specific anti-Stat3 antibody detected two bands; however, in Fig. 1, D and F, it cross-reacted with three bands. The variation in the number of protein bands detected may be due to difference in the expression level of proteins/isoforms at the time of harvesting cells.

The 74–78-kDa Protein(s) Is Phosphorylated at Tyrosine in α-Thrombin-treated Cells—Specific recognition of the 74–78-kDa protein(s) by the phospho-specific anti-Stat3 antibody in α-thrombin-treated cells suggested that it is likely to be phosphorylated at a tyrosine residue(s) by tyrosine kinases. To establish the role of tyrosine kinases, we tested the ability α-thrombin to stimulate tyrosine phosphorylation of the 74–78-kDa protein(s) in the presence and absence of the tyrosine kinase inhibitor genistein. Fig. 2A demonstrates that pretreatment of cells with genistein significantly inhibited the α-thrombin-induced tyrosine phosphorylation. When the blot in Fig. 2A prepared and immunoprecipitated (IP) with phospho-specific anti-Stat3 antibody. Immunocomplexes were collected, run on a 8% gel, and immunoblotted with anti-Stat3 antibody. F, the blot in E was reprobed with phospho-specific anti-Stat3 antibody. These blots are representative of four independent experiments. The position of the 74–78-kDa protein(s) is shown in square brackets. Th, α-thrombin; CR, cross-reactive.

FIG. 1. The phosphospecific anti-Stat3 antibody cross-reacts with 92-kDa Stat3 protein in IL-6-treated cells and a 74–78-kDa protein(s) in α-thrombin-treated cells. A, serum-starved cells were left untreated (control) (lane 1), α-thrombin (0.4 units/ml; 40 min) (lane 2), or treated with IL-6 (20 ng/ml; 15 min) (lane 3), or pretreated first with α-thrombin (0.4 units/ml; 25 min) and then with IL-6 (20 ng/ml; 15 min) (lane 4). These treatment conditions of cells with α-thrombin and IL-6 were the same as our previously published report (20), in which we demonstrated that a 25-min pretreatment of cells with α-thrombin is required to completely inhibit IL-6 (15 min treatment)-induced Stat3 tyrosine phosphorylation. Thus, the total time of α-thrombin exposure of cells representing lanes 2 and 4 was 40 min; whereas the total time of IL-6 exposure of cells representing lanes 3 and 4 was 15 min. Following treatment, total cell lysates were prepared, and proteins were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with phosphospecific anti-Stat3 antibody. B, the blot in A was stripped and reprobed with anti-Stat3 antibody specific to the NH2-terminal region of Stat3. C and D show that interaction of the phospho-specific anti-Stat3 antibody with the 74–78-kDa protein is specific. C, the phosphospecific Stat3 antibody was preincubated with 1 μM Stat3 phospho-peptide (SAAP*Y*LTTFK, where Y* indicates phosphorylated tyrosine) for 2 h at room temperature, and probed with the blot containing protein from α-thrombin-treated cells. D, the phosphospecific anti-Stat3 antibody was preincubated with 1 μM nonphosphorylated Stat3 peptide (SAAPYLTKTF) for 2 h at room temperature, and probed with the blot containing protein from α-thrombin-treated cells. The position of Stat3 is indicated by an arrow. E shows that the 74–78-kDa protein does not co-precipitate with Stat3. The α-thrombin and IL-6 treatment conditions for E was similar to A. Cell lysates were
A, effect of genistein on the ability of α-thrombin to induce tyrosine phosphorylation of the 74–78-kDa protein(s). Serum-starved cells were left untreated (lane 1) or were treated with α-thrombin alone (0.4 units/ml) for 15 min (lane 2), or first pretreated with genistein (20 μM) for 45 min, and then with α-thrombin (0.4 units/ml) for 15 min (lane 3). Cell lysates were prepared, run on a 8% SDS-polyacrylamide gel, and immunoblotted with phospho-specific anti-Stat3 antibody. B, the blot in A was stripped and reprobed with anti-Stat3 antibody. C shows that the 74–78-kDa protein(s) is phosphorylated at the tyrosine residue. The lysates from control and α-thrombin (0.4 units/ml) 15 min-treated cells were immunoprecipitated with anti-phosphotyrosine antibody and immunocomplexes were run on an 8% SDS-polyacrylamide gel and immunoblotted with phospho-specific anti-Stat3 antibody. The position of the 74–78-kDa protein is shown in brackets. These blots are representative of three independent experiments. IgG H, immunoglobulin heavy chain. CR, cross-reactive.

was stripped and reprobed with anti-Stat3 antibody, equal amounts of protein were observed in all lanes (Fig. 2B). To confirm further the 74–78-kDa protein is phosphorylated at tyrosine, proteins from control and α-thrombin-treated cells were immunoprecipitated with anti-phosphotyrosine antibody, and immunocomplexes were run on a gel and immunoblotted with phospho-specific anti-Stat3 antibody. Fig. 2C demonstrates that phospho-specific anti-Stat3 antibody recognized the 74–78-kDa protein(s) in the anti-phosphotyrosine antibody immunoprecipitates with enhanced cross-reactivity in α-thrombin-treated cells. These results clearly establish that the 74–78-kDa protein(s) is phosphorylated at a tyrosine residue(s) in α-thrombin-treated cells.

α-Thrombin-mediated Tyrosine Phosphorylation of 74–78-kDa Protein Occurs via Protease-activated Receptor Type 1 (PAR1)—To determine further the specificity of α-thrombin action, we tested the ability of the thrombin receptor agonist peptide (TRP) (SFLLRN; represents agonist peptide for protease activated receptor 1 or PAR1) (25) to stimulate tyrosine phosphorylation of the 74–78-kDa protein(s). Fig. 3A proves that exposure of cells to both α-thrombin (lane 2) or TRP (lane 3) induced tyrosine phosphorylation of the 74–78-kDa protein(s). The level of induction by TRP was similar to that elicited by α-thrombin, indicating that activation of the α-thrombin receptor was sufficient to induce a full stimulatory effect. Hirudin, an inhibitor of the action α-thrombin, prevented the ability of α-thrombin to stimulate tyrosine phosphorylation (Fig. 3A, lane 4). Reprobing the blot showed a similar amount of Stat3 in all lanes (Fig. 3B). These results show that α-thrombin stimulates tyrosine phosphorylation of the 74–78-kDa protein(s) via PAR1 receptor.

α-Thrombin Rapidly Stimulates Tyrosine Phosphorylation of the 74–78-kDa Protein(s)—To determine the time course of tyrosine phosphorylation of the 74–78-kDa protein(s), we prepared total lysates from untreated or cells treated with α-thrombin for different times, and the samples were immunoblotted with phospho-specific anti-Stat3 antibody. Fig. 3C demonstrates that α-thrombin-induced tyrosine phosphorylation of the 74–78-kDa protein(s) was detected as early as 5 min and reached a maximum at 15 min; however, low levels were also present at 2 h. To confirm equal loading in all lanes of Fig. 3C, we stripped the blot and reprobed with anti-Stat3 antibody. Fig. 3D shows that all lanes contained similar amounts of Stat3 protein. The effect of α-thrombin was also observed at a very low concentration (0.02 unit/ml; data not shown). These results show that α-thrombin rapidly induces tyrosine phosphorylation of the 74–78-kDa protein(s).

The 74–78-kDa Protein Is Localized to the Cytoplasm in Both Unstimulated and α-Thrombin-stimulated Cells—We next sought to determine the cellular localization of the 74–78-kDa protein(s). We isolated total membrane (endoplasmic reticulum, plasma membrane, and mitochondria), cytoplasm, and nuclear fractions from unstimulated and α-thrombin-stimulated cells, proteins immunoblotted with phospho-specific anti-Stat3 antibody. Fig. 4A demonstrates that the 74–78-kDa protein(s) is localized to cytoplasm (Fig. 4A, lanes 3 and 4) and was completely absent in membrane and (lanes 1 and 2) nuclear fractions (lanes 5 and 6). As a control for the quality of cytoplasmic, membrane, and nuclear preparations, the protein samples were also immunoblotted with anti-Stat3 antibody (Fig. 4B), anti-TGF-β-receptor antibody (Fig. 4C), and anti-E2F-1 antibody (Fig. 4D). Since α-thrombin does not induce tyrosine phosphorylation of Stat3 (20), it will not translocate from cytoplasm to the nucleus and therefore is expected only in the cytoplasm of both control and stimulated cells. TGF-β receptor is a plasma membrane protein and therefore is expected only in membrane fractions. Because E2F-1 is a nuclear transcription factor, it is expected to be present only in nuclear fractions. Consistent with this, we detected the presence of Stat3 protein only in the cytoplasmic (Fig. 4B, lanes 3 and 4) and TGF-β receptor in the membrane (Fig. 4C, lanes 1 and 2), and E2F-1 protein in the nuclear fractions (Fig. 4D, lanes 5 and 6). These results clearly prove that the 74–78-kDa protein is localized to the cytoplasmic fraction in both unstimulated and α-thrombin-stimulated cells.

The 74–78-kDa Protein(s) Is Related to Glucose-regulated Protein 75 (GRP-75), a Member of Heat Shock/Stress Response Protein Family—We next performed experiments to establish the identity of the 74–78-kDa protein(s). The strategy used involved immunoprecipitation of the 74–78-kDa protein(s) with phospho-specific anti-Stat3 antibody, followed by SDS-PAGE and mass spectrometry (peptide mass fingerprinting). Mass spectrometry provides the peptide mass and spectral resolution of the peptides generated from the digestion of the
protein with trypsin. Protein identification by this method involves correlation of mass spectrometry data (peptide mass) with theoretical peptide mass data for all known proteins from sequence data bases. For this, lysates from α-thrombin-treated cells were immunoprecipitated with phospho-specific anti-Stat3 antibody, and the resulting protein complexes were electrophoresed on an 8% SDS-polyacrylamide gel and immunobotted with the phosphospecific anti-Stat3 antibody. A, the blot in figure A was stripped and reprobed with anti-Stat3 antibody. C shows the time course of tyrosine phosphorylation of the 74–78-kDa protein(s) by α-thrombin. Serum-starved cells were left untreated or treated with α-thrombin (0.4 units/ml) for different times, and total extracts were prepared, run on a 8% SDS-polyacrylamide gel, and immunobotted with phospho-specific anti-Stat3 antibody. D, the blot in C was stripped and reprobed with anti-Stat3 antibody. These blots are representative of three independent experiments. CR, cross-reactive; TRP, thrombin receptor agonist peptide.

sequence data bases. For this, lysates from α-thrombin-treated cells were immunoprecipitated with phospho-specific anti-Stat3 antibody, and the resulting protein complexes were electrophoresed on an 8% SDS-polyacrylamide gel and then stained with Coomassie Blue. The 74–78-kDa protein(s) bands were excised from the gel, digested with trypsin, and analyzed by mass spectrometry. The masses detected for 21 peptides generated from 74–78-kDa protein(s) matched (20 ppm accuracy window) perfectly with the GRP-75 sequence (GenBank™ accession number U92313), with a Mowse score of 5.785e107.

GRP-75 protein, which is a member of HSP family, has a total of 679 amino acids (26, 27). Put together, the 21 peptides covered 32% of the GRP-75 sequence. Sequence coverage is a measure of total protein sequence represented by the peptide fragments matched in the search result, and coverage of greater than 30% is considered excellent for a pure protein present in high abundance.2 The search was done for all species (in the NCBI database) for a molecular mass range of 5–250 kDa, and the best match was found with hamster GRP-75. All of the other subsequent lower matches were also members of HSP family.

GRP-75 Is Tyrosine-phosphorylated in Cells Stimulated with α-Thrombin—We hypothesized that GRP-75 may undergo tyrosine phosphorylation in cells treated with α-thrombin. To test this, lysates from unstimulated and cells stimulated with α-thrombin were immunoprecipitated with anti-GRP75 antibody, protein complexes separated on a SDS-polyacrylamide gel, and immunobotted with anti-phosphotyrosine antibody. FIG. 5A demonstrates that GRP-75 undergoes tyrosine phosphorylation in α-thrombin-treated cells. It is important to note that although two forms (precursor and mature forms) of GRP-75 are reported to be present in cells (26), we observed only one tyrosine-phosphorylated protein band in FIG. 5A. However, when the blot was stripped and reprobed with anti-GRP-75 antibody, we observed both forms of GRP-75, sug-

\[2 R. G. Cook, personal communication.\]
suggesting that both the precursor and mature forms are immunoprecipitated by the anti-GRP-75 antibody. Fig. 5B confirms equal amount of protein in all lanes.

**Pervanadate, a Stress-inducing Agent, Stimulates Tyrosine Phosphorylation of the 74–78-kDa Protein(s), Whereas Cytokines and Growth Factors Are Ineffective**—In rat hepatoma cells, pp75, a GRP-75 related protein was shown to undergo tyrosine phosphorylation upon treatment with the stress-inducing agent, pervanadate (28). However, heat shock did not stimulate the tyrosine phosphorylation of pp75 (28). We determined if exposure of CCL39 cells with pervanadate and heat shock would induce tyrosine phosphorylation of the 74–78-kDa protein(s). Cells were treated with pervanadate for 15 min or were incubated at 42.5 °C for 30 min, and lysates were immunoblotted with the phospho-specific anti-Stat3 antibody. These blots are representative of four independent experiments.

**DISCUSSION**

The present study is derived from the work we performed earlier on the ability of α-thrombin to negatively regulate IL-6-induced Stat3 signaling in lung fibroblasts (20). In this study, we demonstrate that the phospho-specific anti-Stat3 antibody raised against a phosphopeptide of Stat3 cross-reacted with a 74–78-kDa protein(s) from α-thrombin-treated cells. Thrombin receptor agonist peptide mimicked the actions of α-thrombin, suggesting that α-thrombin mediates this effect through the PAR1 receptor. The newly identified protein was localized to cytoplasm in both unstimulated and α-thrombin-stimulated cells. Immunoprecipitation of the 74–78-kDa protein(s) with phosphospecific antibody and subsequent mass spectrometry identified this protein as a member of heat
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Fig. 7. Amino acid sequence comparison of Stat3 phosphopeptide (immunogen) with GRP-75 shows a common tri-peptide sequence (PYL). The Stat3 phosphopeptide (aa 701–710) was used as immunogen. The tyrosine at 705 in Stat3 undergoes tyrosine phosphorylation in response to IL-6. Note that the GRP-75 also has a PYL motif (aa 330–332), which possibly undergoes phosphorylation in cells treated with α-thrombin.

The interaction of the phospho-specific anti-Stat3 antibody with the 74–78-kDa protein(s) was specific. This is demonstrated by the fact that preincubation of the phosphospecific anti-Stat3 antibody with the Stat3 phosphopeptide abolished its ability to cross-react with the 74–78-kDa protein(s). It is also not a degraded product of Stat3 due to the action of intracellular proteases or exogenously added α-thrombin. This conclusion is supported by the observation that antibodies, which were specific to NH₂, COOH, and internal regions of Stat3, all failed to recognize the 74–78-kDa protein(s); however, these antibodies recognized the 92-kDa Stat3 protein in all immunoblots. This demonstrates that the 74–78-kDa protein is clearly distinct and unrelated to Stat3. These findings suggest that the 74–78-kDa protein contains an antigenic epitope that is tyrosine-phosphorylated in response to α-thrombin, and this is likely to be recognized by the phospho-specific anti-Stat3 antibody.

In order to explain the cross-reactivity of the phospho-specific anti-Stat3 antibody with GRP-75-related 74–78-kDa stress-response protein, we compared the Stat3 phosphopeptide sequence (SAAPY*LKTKF, aa 701–710; where Y* represents phosphotyrosine), which was used as the immunogen, with GRP-75 amino acid sequence. In Stat3, the tyrosine at position 705 was demonstrated to undergo phosphorylation upon treatment of cells with IL-6 (23). Amino acid sequence comparison showed that the PTL tri-peptide sequence (aa 704–706) in the Stat3 phosphopeptide, which is likely to be recognized by the phospho-specific anti-Stat3 antibody, is also present in GRP-75 (aa 330–332) (Fig. 7). Except for the PTL sequence, no other homology was observed between Stat3 peptide and GRP-75. One explanation for the cross-reactivity is that the tyrosine in the tri-peptide PTL of GRP-75-related protein(s) may undergo phosphorylation following treatment of cells with α-thrombin, and this site would then be recognized by the phospho-specific Stat3 antibody. Because antibodies to epitopes outside the phosphorylation domain are removed by extensive pre-adsorption with unphosphorylated peptide during the purification (BIOSOURCE), it is likely that the phospho-specific anti-Stat3 antibody binds to the tri-peptide PYL. It is also to be noted that other HSP70 family members (HSC70, HSP70, and GRP-78) do not have the PTL motif, and consistent with this, the phospho-specific anti-Stat3 antibody failed to cross-react with these family members in immunoblots (data not shown). We also searched the data base for cytoplasmic proteins that may contain a PTL sequence, and hence may undergo phosphorylation in response to α-thrombin. We identified a total of 7 proteins with a PYL sequence in the 74–78-kDa range; however, only two were cytoplasmic (phosphatidylinositol 4-kinase and glycogen-branching enzyme). Although these two proteins contained a PYL sequence, they did not contain any homology to GRP-75 or other HSP members. Therefore, these proteins are unlikely to be the target of tyrosine phosphorylation by α-thrombin.

We observed in many experiments that the 74–78-kDa protein(s) recognized by the phospho-specific Stat3 antibody contains at least three distinct bands (e.g. Fig. 4A, lanes 3 and 4). Deglycosylation experiments showed that it is not due to modification by glycosylation (data not shown). Two possibilities can explain the appearance of these bands: 1) these may arise due to mobility shift caused by phosphorylation of the same protein at multiple sites; 2) they may represent three separate but closely related isoforms phosphorylated at one site. However, in anti-phosphotyrosine blots of immunoprecipitated GRP-75, only one tyrosine-phosphorylated band was observed (Fig. 5A). This suggests that although one of the three bands detected by the phosphospecific Stat3 antibody in Fig. 4A is likely to be GRP-75, the others bands are likely to represent separate but closely related novel isoforms. The GRP-75 gene has 17 exons (29), and therefore, it is also possible that isoforms may arise from alternative splicing. However, until now, alternatively spliced GRP-75 protein products have not been reported. We have demonstrated the phosphorylation of these proteins at the tyrosine residue; however, it is not known at this stage if they are also phosphorylated at serine and threonine. Molecular cloning of these proteins is required to completely establish their identity.

GRP-75 is a member of the HSP family (26, 27). It has also been identified/cloned as mortal-2, peptide-binding protein-74 (PBP-74), and mitochondrial HSP70 (mtHSP70) in various cell types (30–33). The only other closely related protein to GRP-75 identified to date is mortalin-1 (31). Mortalin-1 differs from GRP-75 (mortalin-2) by only two amino acids in the carboxyl terminus, and both are products of separate genes. Despite the high degree of homology, mortalin-1 and GRP-75 are differentially distributed in normal (pancytiosolic) and immortal (perinuclear) mouse cell lines, respectively (31). GRP-75 is induced under conditions of low glucose and other nutritional and environmental stress (31). It is involved in various chaperoning functions in protein translocation, folding, and function in mitochondria (33) and also appears to play a role in antigen recognition (30), cell proliferation, and senescence (31). Moreover, GRP-75 has been shown to inactivate the tumor suppressor/anti-apoptotic protein p53 and abrogates nuclear translocation of wild type p53 (34). Such inactivation of p53 resulted in the down-regulation of p53-responsive genes p21WAF1 and mdm-2. Overexpression of GRP-75 in NIH 3T3 and MRC-5 cells (normal human diploid fibroblasts) led to their malignant transformation and life span extension, and this was shown to be mediated in part by inactivation of p53 (35, 36).

Our results show that the 74–78 kDa, GRP-75-related protein(s) is phosphorylated at tyrosine residues in α-thrombin-treated cells. Although the molecular consequences of this tyrosine phosphorylation are presently unknown, phosphorylation is a key regulatory event in numerous signal transduction pathways and cellular processes (37). Heat shock proteins in general are not considered as targets for protein phosphorylation. Apart from GRP-75 phosphorylation (see Ref. 28 and this study), only phosphorylation of HSP27 (38, 39) and HSP90 (40, 41) have been reported. Phosphorylation of HSP27 and HSP90 appear to have functional consequences. HSP27, which is constitutively expressed in most human cells, undergoes serine...
phosphorylation in response to stress, and this modulates actin filament dynamics (42). HSP90 is serine/threonine-phosphorylated in cells treated with the protein phosphatase inhibitor okadaic acid (43). It was demonstrated that HSP90 associates transiently with pp60  v-Src, directs its cellular trafficking, and negatively regulates its kinase activity. Moreover, serine/threonine phosphorylation of HSP90 and/or pp60  v-Src functions as a regulatory molecular trigger to release pp60  v-Src from the chaperone complex at the inner surface of cell membranes. Recent studies (40, 41) have also shown tyrosine phosphorylation of HSP90. For example, tyrosine phosphorylation of HSP90 within the P2X<sub>7</sub> receptor complex was shown to negatively regulate P2X<sub>7</sub> receptors (41). Taken together, these findings and our results suggest the possibility that phosphorylation of GRP-75-related protein(s) plays a role in α-thrombin-treated cells.

α-Thrombin is a key regulatory molecule produced at the site of tissue injury. It was shown that, within 2 min following ischemia, the levels of glucose and oxygen decrease to nearly undetectable levels (44). This would clearly expose the cells to stress such as hypoxia, hypoglycemia, and oxidative stress, which eventually may lead to cell death (45). Studies in primary cultures of astrocytes and neurons demonstrate that α-thrombin can protect cells from apoptosis induced by hypoglycemia and oxidative stress (45). In CCL39 cells α-thrombin was shown to prevent serum withdrawal induced apoptosis (46). Increased GRP-75 expression has been detected following ischemia in brain (27), and it has been shown to interact with the anti-apoptotic protein p53 (34). It will be interesting to determine whether the tyrosine-phosphorylated 74–78 kDa, GRP-75-related protein(s) plays a role in α-thrombin-mediated prevention of apoptosis through interaction with other cell constituent proteins such as p53. This represents a new area of investigation.

Acknowledgments—We thank Dr. Richard Cook (Baylor College of Medicine, Houston, TX) for Mass Spectrometric analysis and G. M. Venkataraman (Fred Hutchinson Cancer Research Center, Seattle, WA) for helpful discussions.

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