HEF1 is a recently described p130Cas-like docking protein that contains one SH3 domain and multiple SH2 binding motifs. In B cells, HEF1 is phosphorylated by a cytoskeleton-dependent mechanism that is triggered by integrin ligation. However, the induction of HEF1 phosphorylation by G protein-coupled receptors has not been reported. We found that HEF1, but not p130Cas, is tyrosine-phosphorylated following stimulation of the rabbit C1a calcitonin receptor stably expressed in HEK-293 cells. The calcitonin-induced tyrosine phosphorylation of HEF1 increased in a time- and dose-dependent manner. Dibutyryl cAMP and forskolin had little or no effect on HEF1 phosphorylation, and the protein kinase A inhibitor H89 failed to detectably inhibit the response to calcitonin, indicating that the G$_i$/cAMP/protein kinase A pathway does not mediate the calcitonin effect. Pertussis toxin, which selectively blocks G$_i$$_o$ signaling, also had no effect. Increasing cytosolic Ca$^{2+}$ with ionomycin stimulated HEF1 phosphorylation and preventing any calcitonin-induced change in cytosolic calcium by a combination of BAPTA and extracellular EGTA completely blocked the calcitonin-induced tyrosine phosphorylation of HEF1. Phorbol 12-myristate 13-acetate also induced HEF1 tyrosine phosphorylation, and the protein kinase C inhibitor calphostin C completely inhibited both calcitonin- and phorbol 12-myristate 13-acetate-stimulated HEF1 phosphorylation. Calcitonin also induced the tyrosine phosphorylation of paxillin and focal adhesion kinase, and the association of these two proteins with HEF1. Pretreatment with cytochalasin D, which disrupts actin microfilaments, prevented the calcitonin-induced HEF1 and paxillin phosphorylation. In conclusion, the calcitonin-stimulated tyrosine phosphorylation of HEF1 is mediated by calcium- and protein kinase C-dependent mechanisms and requires the integrity of the actin cytoskeleton.

The 130-kDa Crk-associated substrate (p130Cas) and the more recently described human enhancer of filamentation 1 (HEF1, also known as CasL) are focal adhesion-associated proteins (1–3) that, together with Efs/Sin, form a family of multi-domain docking proteins (3–6). Each of these proteins contains an SH3 domain that binds focal adhesion kinase (FAK$^1$ and the structurally related Pyk2 (2, 3, 7–10), a domain rich in SH2-binding sites that are phosphorylated by or associate with a number of oncproteins, including Crk family members, Abl, and Src family tyrosine kinases (3, 8, 11–14), and a highly conserved carboxyl-terminal domain that mediates homo- and heterodimerization of the Cas family members (3). The tyrosine phosphorylation of p130Cas, the first member of the Cas family to be identified, is induced by a number of stimuli, including engagement or ligation of integrins, membrane depolarization, osmotic shock, and activation of multiple types of receptors (e.g. B cell and T cell receptors, receptors for epidermal growth factor, interleukin-8, and urokinase-type plasminogen activator, and the G protein-coupled receptors (GPCR) for lysophosphatidic acid and bombesin) (15–24).

In addition to their localization at focal adhesion sites, several other pieces of evidence suggest that p130Cas and HEF1 may play important roles in cell attachment and motility. Following stimulation of lymphocyte integrins by either cell attachment or ligation with antibodies, both p130Cas and HEF1 are tyrosine-phosphorylated along with other focal adhesion and cytoskeletal proteins, including FAK, paxillin, tensin, and cortactin (12, 15, 16). Expression of p130Cas or its adaptor protein partner Crk promotes cell migration by a mechanism that is dependent on p130Cas tyrosine phosphorylation and the resulting association with Crk (25, 26). In the absence of p130Cas, v-Src and v-Ras-transformed cells exhibit the flat morphological characteristic of untransformed cells rather than the rounded and refractile form of transformed cells (27, 28). Furthermore, bombesin-induced p130Cas tyrosine phosphorylation and the tyrosine phosphorylation of HEF1 induced by integrin ligation in B cells and T cells are inhibited by cytochalasin, which disrupts the network of actin microfilaments (12, 15, 23).

Although HEF1 shares a number of structural and functional characteristics with p130Cas, the two proteins differ in certain respects. Thus, although p130Cas is abundantly expressed in many cells and tissues, HEF1 expression is more variable, being highly expressed in differentiating B and T cells and in tissues that are rich in epithelial cells (3, 4, 15) but not in several other tissues (e.g. heart, brain, and pancreas) (3). In contrast to p130Cas, which is localized primarily at focal adhe-

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1 The abbreviations used are: FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; CT, calcitonin; CTR, calcitonin receptor; PKA, protein kinase A; PKC, protein kinase C; FMA, phorbol 12-myristate 13-acetate; db-cAMP, N6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium; PTX, pertussis toxin; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N,N'-tetraacetic acid; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid tetra(acetoxyethyl) ester.

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**CALCITONIN INDUCES THE ASSOCIATION OF HEF1, PAXILLIN, AND FOCAL ADHESION KINASE**

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Calcitonin Induces Tyrosine Phosphorylation of HEF1

sions and along stress fibers (1), HEF1 is also present in the nucleus and, in some cells, in the Golgi apparatus, and it is observed in large clustered structures in the lamellipodia of HeLa cells (3). During mitosis, a protease-generated aminoterminal fragment of HEF1 associates with the spindle apparatus (29). Furthermore, the Pyk2-dependent phosphorylation of p130Cas but not that of HEF1 requires the presence of Src (9).

It has been reported that p130Cas can be tyrosine phosphorylated by activation of GPCRs. The stimulation of diverse GPCRs, including muscarinic receptors, the lysophosphatidic acid receptor, and the receptors for bombesin and bradykinin, also induces the prominent tyrosine phosphorylation of paxillin and FAK, two other components of focal adhesions plaques that form complexes with p130Cas (23, 30–32). Although some instances of GPCR-dependent tyrosine phosphorylation of p130Cas, paxillin, and FAK reported to date appear to be independent of PKC (24, 30), the activation of PKC can also induce the tyrosine phosphorylation of these proteins (24, 30, 32). The GPCR-induced tyrosine phosphorylation of p130Cas, paxillin, and FAK is typically accompanied by a profound reorganization of actin cytoskeleton, leading to the formation of actin stress fibers and the assembly of focal adhesions (30, 33, 34). To date, HEF1 has been reported to be tyrosine-phosphorylated in response to the activation of integrins, B cell receptors and T cell receptors (9, 12, 15, 35, 36), but in contrast to p130Cas, it has not been shown to be tyrosine phosphorylated by GPCRs.

Calcitonin (CT) is a polypeptide hormone that induces hypocalcemia and has therefore been widely used for the treatment of diseases that are characterized by increased serum calcium, such as osteoporosis, Paget’s disease, and late stage malignancies. The effects of CT are mediated by the CT receptor (CTR), a GPCR that couples to Gs, Gi/o, and Gq (37–41) and regulates kinases (Erk1/2) (40–43). Because we found that a terminal fragment of HEF1 associates with the spindle apparatus (1), HEF1 is also present in the nucleus and, in some cells, in the Golgi apparatus, and it is detected by Western blotting (Fig. 2B) or in 0.5% SDS, and immunoprecipitations were performed with anti-HEF1 number 2. In both cases, the heavy tyrosine phosphorylated (Fig. 1, compare A and B). Finally, to confirm that the tyrosine-phosphorylated 105-kDa protein was HEF1 and not a protein of similar size that binds to and co-immunoprecipitates with HEF1, duplicate sets of untreated and CT-activated cells were lysed with either the non-ionic lysis buffer described under “Experimental Procedures” or in 0.5% SDS, and immunoprecipitations were performed with the anti-HEF1 number 2. In both cases, the heavy tyrosine-phosphorylated 105-kDa protein was selectively removed from the lysate (data not shown), indicating that this protein is indeed HEF1. Thus, CT induces the tyrosine phosphorylation of HEF1 but not the related p130Cas in CTR-expressing HEK-293 cells.

To determine the time course of CT-induced HEF1 tyrosine phosphorylation, C1a-HEK cells were treated with 1 nM CT for increasing periods of time. The cells were lysed, HEF1 was immunoprecipitated with the C/H antibody that recognizes both p130Cas and HEF1 (Fig. 1A). Stripping the membrane and reprobing with anti-C/H revealed two bands, at 105 kDa and 130 kDa, in the lysates and C/H immunoprecipitates and a single 105-kDa band in the HEF1 number 1 immunoprecipitates (Fig. 1B). The 105-kDa bands in the C/H blot corresponded to the 105-kDa band in the anti-P-Tyr blot, indicating that this tyrosine phosphorylated band was HEF1. Similar results were obtained when the immunoprecipitation and immunoblot were performed with anti-HEF1 number 2 (data not shown). Although the anti-C/H antibody immunoprecipitated both p130Cas and HEF1, only HEF1 was detectably tyrosine phosphorylated (Fig. 1, compare A and B).

RESULTS

Calcitonin Induces Tyrosine Phosphorylation of HEF1 but Not p130Cas in C1a-HEK Cells—We initially investigated whether treating C1a-HEK cells with CT would induce the tyrosine phosphorylation of p130Cas and/or HEF1. Treatment of the cells for 3 min with 1 nM CT induced an increase in the tyrosine phosphorylation of proteins of approximate molecular mass = 100–140, 93, 60–70, and 30 kDa (Fig. 1, TLC lanes). The heavily phosphorylated band at 105–110 kDa was immunoprecipitated by anti-HEF1 number 1 and by anti-C/H, an antibody that recognizes both p130Cas and HEF1 (Fig. 1A). Stripping the membrane and reprobing with anti-C/H revealed two bands, at 105 kDa and 130 kDa, in the lysates and C/H immunoprecipitates and a single 105-kDa band in the HEF1 number 1 immunoprecipitates (Fig. 1B). The 105-kDa bands in the C/H blot corresponded to the 105-kDa band in the anti-P-Tyr blot, indicating that this tyrosine phosphorylated band was HEF1. Similar results were obtained when the immunoprecipitation and immunoblot were performed with anti-HEF1 number 2 (data not shown). Although the anti-C/H antibody immunoprecipitated both p130Cas and HEF1, only HEF1 was detectably tyrosine phosphorylated (Fig. 1, compare A and B). Finally, to confirm that the tyrosine-phosphorylated 105-kDa protein was HEF1 and not a protein of similar size that binds to and co-immunoprecipitates with HEF1, duplicate sets of untreated and CT-activated cells were lysed with either the non-ionic lysis buffer described under “Experimental Procedures” or in 0.5% SDS, and immunoprecipitations were performed with the anti-HEF1 number 2. In both cases, the heavy tyrosine-phosphorylated 105-kDa protein was selectively removed from the lysate (data not shown), indicating that this protein is indeed HEF1. Thus, CT induces the tyrosine phosphorylation of HEF1 but not the related p130Cas in CTR-expressing HEK-293 cells.

To determine the time course of CT-induced HEF1 tyrosine phosphorylation, C1a-HEK cells were treated with 1 nM CT for increasing periods of time. The cells were lysed, HEF1 was immunoprecipitated with α-HEF1 number 1, and the immune complexes were analyzed for changes in tyrosine phosphorylation by Western blotting (Fig. 2A). The phosphorylation of HEF1 was detected within 1 min of the addition of CT and reached a maximum by 3 min that was maintained for at least 1 h.

To determine the concentration-dependence of CT-induced HEF1 phosphorylation, cells were treated with increasing...
Calcitonin induces tyrosine phosphorylation of HEF1

Calcitonin stimulates tyrosine phosphorylation of HEF1 but not p130Cas. C1a-HEK cells were treated with 1 nM CT for 3 min and then lysed, and 300 μg of lysates were immunoprecipitated (IP) by 4 μg of either HEF1-specific anti-HEF1 number 1 or anti-C/H, which recognizes both p130Cas and HEF1. Total cell lysates and immunoprecipitated proteins were processed as described under “Experimental Procedures” and immunoblotted with anti-phosphotyrosine (P-Tyr) antibody (A, 1:1000). The membrane was stripped and reprobed with anti-C/H (1:500). B shows the 95–150-kDa region of the C/H blot, aligned with the corresponding region of the phosphotyrosine blot.

amounts of CT for 3 min, then lysed, and processed as above (Fig. 2B). HEF1 phosphorylation was induced by the lowest concentration of CT tested, 0.01 μM, and the maximum effect was achieved with 10 nM CT. Thus, CT induces a strong time- and dose-dependent phosphorylation of HEF1.

Calcitonin-induced Tyrosine Phosphorylation of HEF1 Is Independent of Gs- and Gia/O-dependent Signaling Mechanisms—We then analyzed the signaling pathways involved in the CT-induced tyrosine phosphorylation of HEF1. The CTR couples through Gs, Gi, and Gq (37–40, 44, 45) to induce several signaling events, including the activation of adenylyl cyclase, PKA, and PKC, and an increase in [Ca2+]i (40–42, 46–48). To determine whether the CT-induced HEF1 phosphorylation is mediated by the adenylyl cyclase/PKA pathway, serum-starved C1a-HEK cells were treated with 200 μM forskolin to activate adenylyl cyclase or with 1 mM db-cAMP to stimulate PKA. db-cAMP only slightly increased HEF1 phosphorylation, and forskolin failed to induce any increase in HEF1 phosphorylation (Fig. 3A). In addition, the protein kinase A-specific inhibitor H89 (10 μM) had no effect on CT-stimulated HEF1 phosphorylation (Fig. 3A). Thus, phosphorylation of HEF1 appears to be independent of cAMP and PKA.

We also examined the possibility that PTX-sensitive Gi/o-coupled signaling pathways play a role in the CT-induced HEF1 phosphorylation. Pretreatment with 100–200 ng/ml PTX for 18 h had no effect on CT-induced HEF1 phosphorylation (Fig. 3A). Thus, neither Gs nor Gi/o-coupled mechanisms contribute significantly to the CT-induced HEF1 phosphorylation.

Calcitonin-induced HEF1 Tyrosine Phosphorylation Occurs via a PKC- and Calcium-dependent Mechanism—In addition to activating Gs- and Gi/o-dependent signaling events, the CTR couples to PLC in C1a-HEK cells (39), leading to the activation of PKC and PKC-dependent responses and an increase in [Ca2+]i (40, 41). We therefore examined whether PKC and/or Ca2+ were involved in the mechanism leading to HEF1 phosphorylation (Fig. 3B). Activation of PKC with 100 nM PMA for 10 min strongly stimulated HEF1 phosphorylation. Conversely, the CT-induced HEF1 phosphorylation was completely blocked by pretreating the cells with the PKC inhibitor calphostin C (1 μM), as was the PMA-stimulated HEF1 phosphorylation. The HEF1 phosphorylation was also inhibited in cells incubated overnight with 10 nM PMA to deplete PKC (data not shown).

CT also induces a biphasic elevation of [Ca2+]i, consisting of an initial transient peak resulting from the release of Ca2+ from the intracellular stores and a sustained phase resulting from the influx of extracellular Ca2+ (40, 47, 48). The elevation in [Ca2+]i is necessary for the CT-induced phosphorylation and activation of the MAP kinases Erk 1 and Erk 2 (40). We therefore examined the possible role of [Ca2+]i in the induction of HEF1 tyrosine phosphorylation (Fig. 3C). To investigate the relative contributions of the two components of the calcium response to CT-stimulated HEF1 phosphorylation, we either added 7.5 mM EGTA to the culture 2 min prior to the addition of CT to chelate the extracellular calcium and abolish the calcium influx or preloaded the cells with 50 μM BAPTA-AM for 30 min to block the initial transient phase (40). Both of the treatments partially inhibited the CT-induced HEF1 phosphorylation, and like the effects of these agents on Erk 1/2 phosphorylation, EGTA had a greater effect than BAPTA. The CT-induced increase in HEF1 phosphorylation was completely blocked by the combined application of BAPTA and the brief EGTA exposure or by prolonged (30 min) treatment of the cells with EGTA to deplete intracellular Ca2+ stores, both of which we have shown to abolish all CT-induced changes in [Ca2+]i.
Calcitonin Induces Tyrosine Phosphorylation of HEF1

Calcitonin Induces the Tyrosine Phosphorylation of Paxillin and FAK and Their Association with HEF1—GPCR ligands that induce p130Cas phosphorylation also induce the tyrosine phosphorylation of paxillin and FAK, two other proteins that are involved in adhesion-related signaling complexes (24, 30, 31). We therefore examined whether CT also induced the tyrosine phosphorylation of paxillin and FAK. Treatment of the cells with 1 nM CT for the indicated times and then lysed, and 500 μg of lysate protein was used for immunoprecipitations (IP) with 3 ng of anti-paxillin (A) or 4 μg of anti-FAK (B). The immune complexes were processed for immunoblotting with anti-phosphotyrosine antibody (P-Tyr, upper panels). The blots were then stripped and probed with an antibody against the target antigen in the immunoprecipitation. C, C1a-HEK cells were treated with 1 nM CT for 3 min and lysed. Immunoprecipitation was performed with anti-paxillin, and the immune complexes and the starting lysates were processed for immunoblotting as described under “Experimental Procedures.” The membrane was sequentially blotted with antibodies that recognize HEF1, paxillin, and FAK, with the membrane being stripped after each blot.

The Integrity of the Actin Cytoskeleton Is Necessary for Calcitonin-induced Tyrosine Phosphorylation of HEF1—As noted above, cytochalasin prevents the induction of HEF1 tyrosine phosphorylation by ligation of β1 integrin or B cell antigen receptor on human tonsillar B cells and B cell lines (15), suggesting that HEF1 phosphorylation is dependent on an intact network of actin microfilaments. We therefore determined whether the treatment of C1a-HEK cells with cytochalasin D would affect CT-induced tyrosine phosphorylation of HEF1 and the associated proteins. The cells were pretreated for 2 h with 2 μM cytochalasin D and then stimulated with 1 nM CT for another 3 min. As shown in Fig. 5, cytochalasin D inhibited the CT-induced HEF1 phosphorylation, as well as the phosphorylation of paxillin. Therefore, CT-induced tyrosine phosphorylation of HEF1, like the tyrosine phosphorylation induced by integrin or B cell receptor signaling, requires the integrity of the actin cytoskeleton.

DISCUSSION

p130Cas and the structurally related protein HEF1 are increasingly implicated in signaling events that are involved in the modulation of cell attachment and cytoskeletal function, and it has even been suggested that p130Cas/Crk association may serve as a molecular switch for the induction of cell migration (25). Cell adhesion induces the association of p130Cas...
Calcitonin Induces Tyrosine Phosphorylation of HEF1

with cytoskeletal and focal adhesion proteins (49, 50), and stress fiber organization is abnormal in p130Cas-null fibroblasts (28). Induction of the tyrosine phosphorylation of p130Cas by a number of growth factors and other mitogenic factors requires an intact actin cytoskeleton and parallels an increase in the cellular content of stress fibers (20, 24, 30). In the case of HEF1, integrin ligation induces its tyrosine phosphorylation in B cells and T cells (4, 12, 15, 35) via a mechanism that, at least in B cells, is also disrupted by cytochalasin (15). These indications that p130Cas and HEF1 are involved in the regulation of cell attachment and motility, together with reports that GPCR for bombesin, lysophosphatidic acid, thrombin, and angiotensin induce the tyrosine phosphorylation of p130Cas (23, 24, 30, 51), led us to examine whether CT could induce the phosphorylation of the related focal adhesion proteins p130Cas and HEF1. We found that HEF1 but not p130Cas was strongly tyrosine-phosphorylated in CT-stimulated C1a-HEK cells, demonstrating that, like p130Cas, HEF1 can be phosphorylated in response to the activation of a GPCR. CT also induced the tyrosine phosphorylation of FAK and paxillin and the formation of a complex containing all three of these proteins. The induction of HEF1 phosphorylation and its association with FAK and paxillin is similar to the response of p130Cas to the activation of some GPCRs (24, 30). Our results make it clear, however, that the biologies of p130Cas and HEF1 are not identical. Both proteins are expressed in the C1a-HEK cells, in fact more p130Cas is detected by the C/H antibody that recognizes both proteins, yet only HEF1 appears to be phosphorylated in the CT-treated cells. A similar selective phosphorylation of HEF1 but not p130Cas has been reported in tonsillar B cells following activation of the B cell receptor (15). Furthermore, the GPCR-activated signaling pathways that lead to the phosphorylation of p130Cas and HEF1 seem to differ in some respects. Thus, the CT-induced phosphorylation of HEF1 was completely blocked by the PKC inhibitor calphostin C. In contrast, inhibition of PKC did not reduce the induction of p130Cas phosphorylation by thrombin in HEK-293 cells (24). It is therefore likely that p130Cas and HEF1 fill somewhat different roles, with different stimuli inducing their phosphorylation and incorporation into signaling complexes with FAK and paxillin. The HEK-293 cells, which endogenously express both p130Cas and HEF1, may be an ideal system for characterizing and comparing the function of these two related proteins and the signaling pathways leading to their phosphorylation.

The CTR couples to Gαs, Gαi0, and Gq to activate multiple signaling pathways (10). However, despite recent advances (40, 41, 43), we recently reported that CT induces the phosphorylation and activation of Erk1/2 in C1a-HEK cells (40). The signaling pathways that lead from the CTR to Erk1/2 phosphorylation and to HEF1 phosphorylation share many features. Both responses involve the activation of PKC and an elevation in [Ca2+]i, but are largely independent of adenyl cyclase/CAMP/PKA. In each case, the CT-induced increase in [Ca2+]i apparently synergizes with other signaling events, possibly the activation of PKC, because an ionophore-induced increase in [Ca2+]i, that is quantitatively similar to the CT-induced increase elicits less phosphorylation than CT. In contrast to the Erk1/2 phosphorylation, however, the CT-dependent induction of HEF1 phosphorylation is not detectably PTX-sensitive. Thus, conditions that selectively interfere with the coupling of the CTR to the PTX-sensitive G proteins could inhibit the Erk response but not the HEF1 response.

In conclusion, we have found that CT induces the tyrosine phosphorylation of the p130Cas-like protein HEF1 by a cytochalasin D-sensitive mechanism and the formation of a complex containing HEF1, paxillin, and FAK. The response of these adhesion-related proteins to CT suggests that they may play a role in mediating the ability of CT to induce changes in cell shape and motility.

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