SIGNAL TRANSDUCTION IN RAT1 FIBROBLASTS IN RESPONSE TO EXTRACELLULAR CALCIUM: ROLE OF THE CALCIUM-SENSING RECEPTOR

by

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CERTIFICATE OF APPROVAL

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This thesis is dedicated to

my grandparents,

Leroy and Irene White

"Thus says the LORD who made the earth, the LORD who formed it to establish it, the LORD is His name, 'Call to Me, and I will answer you, and I will tell you great and mighty things, which you do not know.'"

Jeremiah 33:2-3

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ABSTRACT

Changes in extracellular calcium (Ca^{2+}_o) concentration can modify the behavior of a number of cell types. For instance, primary keratinocytes proliferate when cultured in low Ca^{2+}_o-containing medium. When the Ca^{2+}_o concentration is raised to 1 mM, however, proliferation of keratinocytes is inhibited and differentiation is triggered. Conversely, primary fibroblasts are quiescent in low Ca^{2+}_o-containing medium but proliferate freely in the presence of elevated Ca^{2+}_o A proximal cellular event in response to elevation of Ca^{2+}_o in both keratinocytes and fibroblasts is increased tyrosine phosphorylation. Specifically, the amount of tyrosine phosphorylated p62 associated with the Ras GTPase activating protein (GAP) is increased in these cells following the elevation of Ca^{2+}_o. Changes in the tyrosine phosphorylation of GAP-associated p62 appear to be linked with changes in GAP activity, thereby influencing the activity of Ras. The ability of multivalent cations to mimic elevations in Ca^{2+}_o , whereas ionophores cannot, suggests that the transducing molecule is a polyvalent cation receptor.

One possible candidate for such a receptor is the recently described calcium-sensing receptor (CaSR). This seven-transmembrane domain receptor is capable of sensing millimolar changes in Ca^{2+}_o and has a physiological role in calcium homeostasis. Little is know about the intracellular consequences of receptor activation, however.

The focus of this study is the potential interactions between the calcium-sensing receptor (CaSR) and pathways involving tyrosine phosphorylation in Rat1 fibroblasts. The effect of the CaSR on Ca^{2+}_o-

stimulated tyrosine phosphorylation was assessed in Rat1 cells stably transfected with the wild-type CaSR or a non-functional mutant. We report here a role of the CaSR in Ca^{2+}_o-dependent pathways involving tyrosine phosphorylation. Specifically, we have observed a Ca^{2+}_o-dependent increase in Src kinase activity, Shc tyrosine phosphorylation, and association of tyrosine phosphorylated p62 with Ras-GAP. Each of these Ca^{2+}_o-dependent changes was abrogated in the presence of a non-functional CaSR, strongly implicating this receptor as a major protein transducing the effects of Ca^{2+}_o on proliferation-associated pathways.

INTRODUCTION

Extracellular calcium (Ca^{2+}_o) plays a pivotal role in signaling pathways leading to growth and differentiation in a number of cell types. Studies in the keratinocyte model suggest that the proximal event in Ca^{2+}_o -mediated differentiation is tyrosine phosphorylation. An immediate increase in tyrosine phosphorylation is also observed in cultured fibroblasts when proliferation is stimulated by elevation of extracellular calcium. Specific cellular mechanisms through which this extracellular signal is transduced have not been fully elucidated. The overall goal of this study is to identify some of these Ca^{2+}_o-sensitive transduction mechanisms in Rat1 fibroblasts.

I. The Calcium-Sensing Receptor

Calcium Homeostasis

Regulation of extracellular calcium levels is a critical function for the well-being of higher eukaryotes. In mammals this ion has important roles in excitation-contraction coupling in muscle, action potentials in nervous tissue, blood clotting, and plasma membrane integrity (reviewed in Brown, Vassilev et. al., 1995; Brown, 1991). Intracellular calcium also serves as a second and third messenger in many signal transduction cascades, including those leading to proliferation and cell differentiation.

Systemic calcium homeostasis is maintained through the combined actions of the thyroid, intestine, bone, and kidneys (reviewed in Brown and Leboff, 1986; Brown, 1991). Decreases in plasma Ca^{2+} levels are sensed by the parathyroid which quickly responds with an increase in parathyroid hormone (PTH) secretion (Blum, Treschel et. al., 1983; Brown, Leombruno et. al., 1985; Wallfelt, Lindh et. al., 1988). PTH subsequently results in renal Ca^{2+} readsorption and enhances the release of this ion from bone reserves via

osteolysis (Aurbach, Marx et. al., 1985). Within the kidney, PTH stimulates the production of vitamin D_3 hormone which assists adsorption of Ca^{2+} from the intestine. This hormone also inhibits the secretion of PTH from the parathryoid, thus acting as a negative feedback regulator.

Increases in extracellular Ca^{2+} are sensed by the C-cells of the thyroid which then release calcitonin. Juxtaposed to PTH, calcitonin decreases renal tubular reabsorption of Ca^{2+} and promotes skeletal uptake of this ion by inhibiting osteoclast activity.

Abnormalities in these calcium-sensing mechanisms can result in severe pathophysiologies (Law and Heath, 1985; Marx, Attie et. al., 1981; Marx, Lasker et. al., 1986; Pollak, Brown et. al., 1993 *and references therein*). Adenomas of the parathyroid and familial hypocalciuric hypercalcemia (FHH) are associated with elevated PTH and hypercalcemia. Symptoms of these diseases include polyuria, polydipsia, and neurological disfunctions. Other inherited forms of hypercalcemia, such as neonatal severe hyperparathyroidism (NSHPT), are often fatal unless parathyroidectomy is carried out within a year after birth.

Abnormalities in vitamin D hormone production, as in the case of rickets and chronic renal disease, is the major cause of secondary hyper-parathyroidism. Since vitamin D hormone is a major inhibitor of PTH production, the absence of this hormone allows for continuous PTH release. The long-term effect of this is decalcification of the bones, ultimately resulting in skeletal damage.

Obviously, the ability of the parathyroid to regulate extracellular Ca^{2+} requires an appropriate Ca^{2+}-sensing mechanism. Evidence supporting the existence of a specific Ca^{2+}-sensing receptor was first obtained by

electrophysiological studies in parathyroid cells. Edward Brown and collegues postulated such a receptor based on observations that the addition of Ca^{2+}, Mg^{2+}, Gd^{3+} and other polyvalent cations to rat parathyroid cells could induce depolarization (reviewed in Brown, Pollak et. al., 1995b; Brown, 1991; Nemeth, 1990). This effect was dose dependent and could not be explained by a simple cation gradient, ie. electromotive force. The addition of Ca^{2+} to these cells also results in a pertussis toxin-sensitive increase in IP_3 production and a concommitant inhibition of cyclic adenosine monophosphate (cAMP) accumulation (Chen, Barnett et. al., 1989). Furthermore, this G-protein inhibitor is known to uncouple Ca^{2+}-sensitive PTH release in these cells, as would be expected for a receptor-mediated mechanism (Fitzpatrick and Aurbach, 1986).

Cloning and characterization of the CaSR.

To facilitate the cloning of this postulated receptor, Brown et. al. utilized an oocyte expression paradigm (Brown, Gamba et. al., 1993). Xenopus oocytes are routinely used as an expression cloning system as they efficiently translate microinjected mRNA. The addition of G-protein coupled receptor mRNA to oocytes results in increased IP_3 production upon ligand stimulation. The subsequent pertussis toxin-sensitive Ca^{2+} mobilization produces large inward Cl- currents, which are then monitored electrophysiologically.

When poly(A)$^+$ RNA from bovine parathyroid was injected into oocytes, an inward Cl^- current was observed in response to extracellular Gd^{3+} (Racke, Dubyak et. al., 1991; Shoback and Chen, 1991). Using this as an assay, the functional screening of a directional cDNA library using the presence of a Gd^{3+}-stimulated Cl^- current as the endpoint led to the isolation of the bovine

parathyroid Ca^{2+}-sensing receptor (BoPCaR1) mRNA (Brown, Gamba et. al., 1993).

Expression of BoPCaR1 mRNA in oocytes resulted in a large Cl^- current in the presence of Ca^{2+}, Gd^{3+} and other polyvalent cations. The affinity of the BoPCaR1 for these ions was remarkably similar to those observed for the native receptor (EC_{50}'s: Ca^{2+} = 3mM, Gd3+ = 60μM for BoPCaR1 compared with 3mM and 70μM, respectively, for the native receptor). The Gd^{3+}-induced current was observed even with the addition of the Ca^{2+} chelator EGTA, indicating that the BoPCaR1 protein is not a Ca^{2+} channel or transporter. All currents were inhibited by pertussis toxin, analogous to what is observed in parathyroid cells.

Northern hybridization of poly $(A)^+$ RNA from bovine tissues using BoPCaR1 as a probe revealed detectable transcripts in the brain, parathyroid, thyroid, and kidney (Brown, Gamba et. al., 1993). This distribution is consistent with the tissues responsible for calcium homeostasis or those known to be most sensitive to Ca^{2+}_o. Based on this tissue distribution and the pharmacological similarities of BoPCaR1 with the native parathyroid receptor, Brown and colleagues have proposed that BoPCaR1 provides the essential external calcium-sensing mechanism for systemic Ca^{2+} homeostasis.

The BoPCaR1 cDNA has a 3255 bp open reading frame. Hydropathy analysis predicts a 7 transmembrane (7TM) receptor with a very large (613 amino acid) acidic extracellular domain. The receptor lacks known high-affinity Ca^{2+} binding domains such as EF hands or carbohydrate binding sites (Baimbridge, Celio et. al., 1992; Clapman, 1995; Drickamer, 1993 *and references therein*). These high affinity sites are present on enzymes such as calmodulin and PKC which are regulated by cytosolic levels (micromolar or less) of Ca^{2+}. In the parathyroid, however, a Ca^{2+} binding affinity on the order of

millimolar would be expected for a receptor which sensed plasma Ca^{2+} levels.

Brown and collegues suggest that the acidic residues in the N-terminal domain of the BoPCaR1 may function as low affinity binding sites for polyvalent cations. The authors cite similarites with the calcium binding protein calsequestrin, which has an affinity for Ca^{2+} of approximately 1mM (Fliegel, Ohnishi et. al., 1987). Although the specific conformational structure of this molecule is not known, acidic residues are present in the calcium binding regions.

The predicted 1085 amino acid protein shares little sequence homology with other proteins in current databases. The highest degree of similarity (~25%) is with the metabotropic glutamate receptors (mGluR's) (Nakanishi, 1992; Tanabe, Masu et. al., 1992). Regions of shared homology include the large extracellular domain and the first and third intracellular loops. The extracellular domains of both these receptors share structural similarity with a family of bacterial periplasmic proteins which also bind inorganic ions (Adams and Oxender, 1989). In the BoPCaR1 the intracellular loops contain two putative protein kinase C (PKC) phosphorylation sites. These sites may play a role in receptor-G protein interactions, and are discussed below.

The calcium sensing receptor (CaSR) has subsequently been cloned from human, rat, and mouse libraries (Ho, Conner et. al., 1995; Pollak, Brown et. al., 1993; Riccardi, Park et. al., 1995; Ruat, Molliver et. al., 1995; Ruat, Snowman et. al., 1996). In one study, Ruat et al examined the location of the CaSR in brain tissues (Ruat, Molliver et. al., 1995). In nervous tissue, depolarization causes Ca^{2+} to influx into nerve terminals, thereby triggering neurotransmitter release. This influx is associated with a decrease in extracellular levels of Ca^{2+}in the synaptic space. Conceivably, to ensure adequate Ca^{2+}_o homeostasis in the synapse, a CaSR-like mechanism could

participate in the maintenance of synaptic Ca^{2+}_o levels. Using a polyclonal antibody raised against an extracellular region of the CaSR, Ruat et. al. localized the protein to nerve terminals. Their findings suggest a function of the CaSR in tissues not directly involved in systemic Ca^{2+} homeostasis and suggest that the CaSR may have broader roles in other Ca^{2+}_o -sensitive cellular processes. It is possible that the CaSR may act as a receptor for this extracellular signal in cell types known to be sensitive to changes in Ca^{2+}_o.

G Protein-Coupled Receptors.

In eukaryotic cells the plasma membrane serves as an impermeable barrier to ions, polar molecules, and most macromolecules. Signaling molecules such as neurotransmitters, hormones, and other cytokines must then rely on cell-surface receptors to influence their target cells. Upon ligand binding these receptors convert the extracellular signal to an intracellular event where it can produce a variety of responses. Among these effects are channel opening, secretion, neurotransmission, and more long-term responses such as induction of gene transcription, chemotaxis, proliferation, and differentiation.

Among the best studied of these receptors is the superfamily of G-protein coupled receptors, also known as 7 transmembrane (7TM) receptors (reviewed in Berridge, 1993; Strader, Fong et. al., 1994; Taylor and Marshall, 1992). As the former name suggests, the intracellular effector common to all members of this family are the heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). Binding with the ligand causes a conformational change in the receptor which allows it to interact with the G-protein on the cytosolic face of the plasma membrane. This receptor-G protein interaction promotes the disassociation of the G-protein complex into

its α and βγ subunits. The α and βγ subunits then modulate the activity of a host of other proximal signaling proteins, including adenylyl and guanylyl cyclases, phospholipases, and ion channels.

Hydropathy, immunofluorescence, and protease analyses suggest that the 7TM receptor family have common structural similarities (reviewed in Ostrowski, Kjelsberg et. al., 1992; Strader, Fong et. al., 1994). All of these receptors share a glycosylated N-terminal extracellular domain, a hydrophobic region which spans the membrane seven times, and a cytoplasmic tail. The seven hydrophobic streches of 20-25 amino acids are predicted to form α-helices, connected by alternating intracellular and extracellular hydrophilic loops. Functional studies have demonstrated a role for these extracellular loops in assisting the N-terminal domain with ligand binding, while the intracellular loops are involved in the process of G-protein coupling.

The third intracellular loop is of particular importance for regulation of signaling pathways by 7TM receptors. In rhodopsin, proteolytic digestion of this region inhibits light-dependent coupling of the receptor to the G-protein transducin (Kuhn and Hargrave, 1981). Similarly, large deletions in the third intracellular loop of the β-adrenergic receptor (β-AR) results in the complete loss of ligand stimulated cAMP production (Dixon, Sigal et. al., 1987).

In many 7TM receptors the third intracellular loop and cytoplasmic tail are rapidly phosphorylated upon ligand binding. In rhodopsin, the β-AR, the follitropin (FSH) receptor, and undoubtedly many others, phosphorylation of the third intracellular loop disrupts the coupling of the receptor to its G-protein effector (Hausdorff, Bovier et. al., 1989; Hausdorff, Caron et. al., 1990; Lefkowitz, 1993; Quintana, Hipkin et. al., 1994 *and references therein*). The net result of this negative feedback loop is that the receptor is no longer able to transduce the extracellular signal, even in the presence of continued ligand

stimulation. This phenomenon is refered to as desensitization, or quenching in the case of rhodopsin (reviewed in Hausdorff, Caron et. al., 1990; Lefkowitz, 1993).

Although there is a plethora of ligands for 7TM receptors, many have common downstream effectors. Chief among these is the inositol trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores (reviewed in Berridge, 1993; Bootman and Berridge, 1995; Taylor and Marshall, 1992). Following receptor stimulation and subsequent G-protein activation, IP_3 and diacylglycerol (DAG) are produced as a result of phosphatidyl inositol hydrolysis by phospholipase C. Accumulation of IP_3 in turn activates the release of Ca^{2+} from intracellular stores as a direct consequence of binding with IP_3 receptors associated with those stores. Receptor activation, therefore, can often be monitored indirectly by measuring increases in intracellular IP_3 or Ca^{2+} levels.

Naturally occurring mutations in the CaSR.

Abnormalities in extracellular Ca^{2+} and PTH regulation can result in severe clinical diseases (reviewed in Brown, 1991). Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant condition characterized by elevated circulating Ca^{2+} and abnormal PTH levels (Law and Heath, 1985; Marx, Attie et. al., 1981). A related syndrome, neonatal severe hyperparathyroidism (NSHPT), is generally fatal unless the parathyroid is removed early in life. These diseases have both been mapped to the same chromosomal locus, 3q2 (Chou, Brown et. al., 1992). Pedigree analysis further demonstrated that NSHPT is the homozygous form of FHH (Pollak, Chou et. al., 1994).

Given the potential role of the CaSR in Ca^{2+} homeostasis and PTH

secretion, Pollak et. al. postulated that mutations to the CaSR gene may be genetically linked to these hypercalcemias (Pollak, Brown et. al., 1993). In support of this, they demonstrated that the CaSR hybridized with chromosome 3 hybrid DNA on Southern blots, consistent with the 3q2 locus. Using ribonuclease protection assays, the group then identified three mutations in the CaSR that segregated with the disease status in FHH family members (Pollak, Brown et. al., 1993). Futhermore, an individual diagnosed with NSHPT had mutations on both alleles of the CaSR gene, again corroborating the pedigree analysis.

One of the mutations identified in their study, the family J mutation, was tested for functionality in expression studies. This mutation (R796W) was recombinantly introduced into the BoPCR1 cDNA and the resulting cRNA was microinjected into oocytes. Compared to the wild-type CaSR, oocytes injected with the R796W cRNA exhibited one tenth the Ca^{2+} - stimulated Cl^- current when treated with Ca^{2+} and other polyvalent cations. In a subsequent study, the coexpression of the R796W mutant receptor with the wild-type CaSR caused a shift to the right in the dose response to Ca^{2+}_o, as monitored by release of intracellular calcium (Bai, Quinn et. al., 1996). These results, combined with the genetic analysis data, suggest that the R796W mutation produces a non-functional receptor, capable of interfering with the normal CaSR when coexpressed.

It should also be noted that this particular mutation lies within a putative PKC consensus sequence in the third intracellular loop of the receptor (Brown, Gamba et. al., 1993). Phosphorylation of this loop by PKC and other serine-threonine kinases inhibits the coupling of many 7TM receptors to G-proteins and associated signaling pathways. (Hausdorff, Bovier et. al., 1989; Hausdorff, Caron et. al., 1990; Lefkowitz, 1993; Quintana, Hipkin

et. al., 1994 *and references therein*). In parathyroid cells, activation of PKC by phorbol esters is known to stimulate PTH secretion (Brown, Redgrave et. al., 1984; Membreno, Chu et. al., 1989). It is quite possible that PKC may exert its influence on PTH release by phosphorylation of the CaSR at R796 and other putative PKC consensus sites with subsequent attenuation in receptor-effector interactions. This model is consistent with the family J disease status and the decreased Cl^- current observed in oocytes injected with R796W mRNA described above.

In addition to the R796W interfering mutant, activating mutants of the CaSR also have adverse effects on calcium homeostasis. Hypocalcemia, or sub-normal levels of serum Ca^{2+}, is often associated with the inability of the parathyroid gland to effectively sense circulating calcium levels. Through pedigree analysis, Pollak et al have identified a mild-form of hypocalcemia that is inherited as an autosomal dominant phenotype (Pollak, Brown et. al., 1994). One family affected with this particular form was screened for mutations to the CaSR by RNase protection assays. A single base change was identified, corresponding to a Glu to Ala (G128A) change in the extracellular domain of the protein.

Oocytes microinjected with CaSR mRNA bearing this mutation had greater amounts of IP_3 accumulation compared to wild type, regardless of extracellular Ca^{2+} concentration (Pollak, Brown et. al., 1994). The presence of this activating mutant in the affected family members, combined with the oocyte data led the authors to conclude that the G128A 'activating' mutation causes this form of hypocalcemia, presumably by increasing the affinity of the receptor for Ca^{2+}_o and enhancing its activity at low or normal serum calcium levels.

CaSR Knockout Mice

Perhaps the best evidence supporting a requirement for the CaSR in Ca^{2+}_o homeostasis comes from transgenic mice studies. Using standard homologous recombination methods, Ho et. al. have disrupted the murine CaSR gene (Ho, Conner et. al., 1995). ES cells selected for the inactive form of the receptor were injected into a mouse blastocyst and reimplanted into the foster mother. The resulting chimeric offspring were crossed with Black Swiss mice and transgenic offspring were identified by southern blot analysis. Heterozygous mice ($CaSR^{+/-}$) were then mated to produce homozygous offspring ($CaSR^{-/-}$).

Relative protein levels of the CaSR in the wild-type, $CaSR^{+/-}$, and $CaSR^{-/-}$ mice were determined by Western blot analysis using a polyclonal antibody raised against the extracellular domain of the receptor (peptides 215-237). Heterozygous mice had receptor levels approximately 50% of wild-type, while CaSR protein was not detectable in the homozygotes. Similar differences in the level of expression were observed by immuno-histochemistry in parathyroid tissues from these strains.

The resultant phenotypes of the $CaSR^{+/-}$ and $CaSR^{-/-}$ mice were strikingly similar to the disease states in humans bearing inactivating mutations in the CaSR. Heterozygous mice were indistinguishable from their wild-type siblings in physical appearance and activity. However, both serum Ca^{2+} and PTH levels were elevated in the $CaSR^{+/-}$ mice, demonstrating a deficiency in their inverse regulation. These same features are common in humans diagnosed with FHH, as described above.

The phenotype for the homozygous mice is severe. $CaSR^{-/-}$ mice exhibit parathyroid hyperplasia, skeletal deformities, reduced body weight and died between 3 and 30 days after birth. These characteristics are analogous to

those observed in patients diagnosed with NSHPT.

In the normal parathyroid, stimulation of the calcium sensing receptor by Ca^{2+} inhibits PTH secretion. Based on the relative protein levels in the different transgenic lines, the authors concluded that the number of functional CaSR receptor molecules on the cell surface is critical in systemic Ca^{2+} homeostasis (Ho, Conner et. al., 1995). Under this model, reduced levels of the CaSR would not be sufficient to completely inhibit PTH release by normal CaSR-mediated signaling pathways. It is conceivable, then, that individuals affected with FHH may lack this critical number of receptors when non-functional receptors (such as the R796W mutant) are expressed (Pollak, Brown et. al., 1993).

Intracellular Effectors of the CaSR in Parathyroid Cells.

As noted, an inverse relationship exists between serum calcium levels and PTH release in parathyroid cells. While the CaSR is directly implicated in this event, relatively little is known about the intracellular mechanisms of CaSR-mediated PTH secretion.

The addition of calcium to cultured parathyroid cells has long been associated with an increase in intracellular calcium concentration $[Ca^{2+}]_i$ (Nemeth and Scarpa, 1986; Shoback, Thatcher et. al., 1983). Similarly, activation of the CaSR by high calcium (greater than 1 mM) and other polyvalent cations also results in an IP_3-mediated release of calcium from intracellular stores (Brown, Chen et. al., 1989). This $Ca^{2+}{}_o$-stimulated increase in $[Ca^{2+}]_i$, however, does not appear to be required for the decrease in PTH secretion (reviewed in Brown, 1991). For example, the addition of ionomycin to parathyroid cells cultured in low calcium (0.5mM) fails to produce an inhibition of PTH release despite a two-fold increase in $[Ca^{2+}]_i$ (Nemeth,

Wallace et. al., 1986). Furthermore, polyvalent cations which are known to activate the CaSR are able to produce a maximal inhibition of PTH release in these cells, but with considerably smaller increases in $[Ca^{2+}]_i$ than are observed in response to elevation of $[Ca^{2+}]_o$. Thus, elevations in $[Ca^{2+}]_i$ may not be sufficient for inhibition of PTH secretion.

Several reports also implicate cAMP levels in the regulation of PTH release. The addition of the cAMP analogues such as dibutyryl cAMP to cultured parathyroid cells is sufficient to induce PTH release (Brown, Gardner et. al., 1978). Similarly, lowering of $[Ca^{2+}]_o$ results in an accumulation of cAMP in cultured parathyroid cells (Brown, Gardner et. al., 1978). This suggests a model where activation of adenylate cyclase by low $[Ca^{2+}]_o$ stimulates PTH secretion. As systemic calcium levels returned to normal, cAMP production would then be predicted to be negatively regulated by the Gi-coupled CaSR.

A model in which the concentration of cAMP is the sole regulator of PTH secretion does not hold up under close experimental scrutiny, however. Elevation of extracellular calcium produces only a modest lowering of cAMP levels; not enough to account for the inhibition of PTH secretion (Brown, Gardner et. al., 1978). Moreover, elevation of $[Ca^{2+}]_o$ has minimal effect on cAMP-dependent protein kinase (PKA) activity (Brown and Thatcher, 1982; Lasker and Spiegel, 1982). These observations suggest that changes in cAMP concentration may not be sufficient to account for all the downstream effects of CaSR activation (Brown, 1991). Other signaling events involved in the regulation of PTH secretion by the CaSR have not yet been characterized.

Signaling Pathways Affected By The CaSR in Non-Parathyroid Cells.

Although the CaSR is implicated in systemic calcium homeostasis,

very few studies to date have investigated the potential role of the CaSR in non-parathyroid cells. As mentioned, Ruat et al have cloned a CaSR from rat nervous tissue and have localized the receptor to nerve terminals (Ruat, Molliver et. al., 1995). An endogenous CaSR has also been reported in the AtT-20 pituitary cell line. By RT-PCR analysis, Emanuel and co-workers identified a 383 base pair fragment from these cells that exhibited 100% sequence identity with previously cloned mouse CaSR cDNA (Emanuel, Adler et. al., 1996). Activation of the CaSR by Gd^{3+}, neomycin or high calcium in AtT-20 cells resulted in pertussis toxin (PTX)-sensitive increase in IP_3 production.

Unlike the situation in parathyroid cells, however, the addition of calcium and gadolinium to AtT-20 cells caused an *increase* in cellular cAMP levels. Additionally, the $Ca^{2+}{}_o$ -stimulated production of cAMP was not affected by pretreatment with PTX. These data suggest that the CaSR can influence intracellular events by both PTX-sensitive and PTX-insensitive pathways, depending on the cell type.

Finally, Ruat and co-workers have observed an elevation of arachidonic acid (AA) release following activation of the CaSR in chinese hamster ovary (CHO) cells (Ruat, Snowman et. al., 1996). Wild-type CHO cells had little IP_3 production or AA release when stimulated with agonists of the CaSR. CHO cells stably transfected with the CaSR, however, showed a large increase in both $Ca^{2+}{}_o$-stimulated IP_3 production (5 fold) and AA release (10-15 fold). IP_3 production is well documented as a response to activation of 7TM receptors. Phospholipase A2, which generates AA, can be activated by IP_3-mediated increases in $[Ca^{2+}]_i$ (Clark, Schievella et. al., 1995; Clark, Lin et. al., 1991; Stephenson, Manetta et. al., 1994). The authors therefore explained the increase in AA release as a downstream event resulting from the CaSR-

stimulated increase in $[Ca^{2+}]_i$.

Role of Ca^{2+} in signal transduction

The importance of Ca^{2+} in cell function has been studied primarily with respect to its role as an intracellular second messenger. Receptor-mediated increases in intracellular Ca^{2+} are known to modulate a number of downstream molecules, including protein kinases and phospholipases. Chief among the protein kinases are the Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) and the Ca^{2+} and the phospholipid-dependent protein kinase C (PKC) families (Dedman, 1984; Huang, 1989; Nishizuka, 1984; Nishizuka, 1992 *and references therein*). In the case of CaM kinase, elevation of intracellular Ca^{2+} triggers its association with calmodulin and subsequent activation (reviewed in Soderling, 1990). For PKC, elevation of intracellular Ca^{2+} causes a conformational change in the protein itself which ultimately results in an activation of the kinase domain. Downstream substrates of these kinases include enzymes that regulate cellular processes including glycolysis, transcription, and protein synthesis (reviewed in Cohen, 1992; Huang, 1989; Karin and Smeal, 1992). Thus, a single extracellular signal can be amplified to influence a Ca^{2+}-sensitive network of signaling cascades.

Changes in extracellular Ca^{2+} levels are also important for normal cellular function. Boyton and his co-workers have demonstrated in a number of epithelial cell types that both the G_o to G_1 and G_1 to S transitions are dependent on extracellular Ca^{2+} concentration; neither transition can occur in Ca^{2+}-free media, and proliferation is blocked (Boyton, 1988). This dependence of cell cycle progression on external Ca^{2+} appears to be common to a number of cell types including keratinocytes, epithelial cells, and fibroblasts.

Role of Ca^{2+}_o in keratinocytes

Extensive studies have been conducted on the regulation of keratinocyte growth and differentiation by extracellular calcium (Boyce and Hamm, 1983; Dykes, Jenner et. al., 1982; Hennings, Holbrook et. al., 1983; Hennings, Michael et. al., 1980; Stanley and Yuspa, 1983). Primary keratinocytes proliferate freely when cultured in low Ca^{2+}-containing medium (.05 to .1 mM). In this medium these cells grow as a monolayer and can be passaged for several months. When the Ca^{2+} concentration is raised to normal plasma levels (1.2-2 mM), however, proliferation of keratinocytes is inhibited and differentiation is triggered. This Ca^{2+}-induced shift to terminal differentiation is associated with increased cell-to-cell contacts, desmosome formation, cornification, and concommitant decrease in DNA synthesis.

Although *in vitro* models often fall short of physiological systems, it appears that culture of keratinocytes closely reflects *in vivo* events. In the epidermis, proliferation is predominant in the lower basal layers and differentiation is favored in the outermost granular regions. Based on the sensitivity of differentiation to media Ca^{2+} concentration in tissue culture, Elias and co-workers postulated that Ca^{2+} may also influence differentiation *in vivo* (Menon, Grayson et. al., 1985).

This hypothesis was tested using an ion capture method for determining relative levels of calcium within the epidermis. The rationale of this cytochemical technique is to precipitate free Ca^{2+} with oxalate. These insoluble complexes are electron dense, and therefore represent Ca^{2+} localization on electron micrographs.

The distribution of dense regions in mouse epidermis indicated low Ca^{2+} localizations in the basal and spinous layers with increased

concentration through the outer granular and stratum granulosum regions. This calcium gradient is consistent with the ion having a role in both proliferation and terminal differentiation in the epidermis. Thus, Ca^{2+}-induced differentiation in cultured keratinocytes appears to correlate well with this event in vivo.

Role of Extracellular Calcium in Other Cell Lines

Extracellular Ca^{2+} concentration also regulates the growth of human breast epithelial cells (BEC's) in culture in a manner similar to keratinocytes (MacGrath and Soule, 1984; Ochieng, Tahin et. al., 1991; Soule, Maloney et. al., 1990). In low Ca^{2+}-containing medium, BEC's proliferate and maintain a ductlike appearance. If this level is raised to 1.05mM or above, the cells undergo terminal differentiation and have a flattened morphology (Ochieng, Tahin et. al., 1991; Soule, Maloney et. al., 1990). This elevation in extracellular Ca^{2+} produces an increase in intracellular Ca^{2+} and activation of associated signaling pathways (Ochieng, Tahin et. al., 1991).

Interestingly, BEC's which are spontaneously immortalized in this high Ca^{2+} media have very low intracellular Ca^{2+} levels compared to their normal counterparts (Ochieng, Tahin et. al., 1991). Apparently, these immortalized cells have either acquired a calcium buffering capacity, or have failed to release Ca^{2+}_i in response to Ca^{2+}_o , thereby allowing for unperturbed growth in a high $[Ca^{2+}]_o$ enviroment.

In contrast to keratinocytes and BEC's, elevation of Ca^{2+}_o stimulates the growth of fibroblasts (Huang, Maher et. al., 1995). Human fibroblasts are quiescent when cultured in low calcium containing media (0.1mM) (Huang, Maher et. al., 1995; Morgan, Yang et. al., 1991). When human fibroblasts are cultured in low Ca^{2+}_o and then stimulated with 1mM calcium, they had

growth curves similar to cells treated with either EGF or serum (Huang, Maher et. al., 1995). Thus, elevation of Ca^{2+}_o acts as a strong mitogen in human fibroblasts.

II. Tyrosine Phosphorylation in Signal Transduction

Tyrosine Phosphorylation in Keratinocyte Differentiation.

In the keratinocyte model, an early and specific event in calcium-induced differentiation is tyrosine phosphorylation. Keratinocytes that are cultured in low Ca^{2+} containing media, and then stimulated with high Ca^{2+}, show a pronounced increase in a number of tyrosine phosphorylated bands when lysates are immunoblotted with anti-phosphotyrosine (anti-PY) antibodies (Calautti, Missero et. al., 1995; Filvaroff, Calautti et. al., 1992; Filvaroff, Calautti et. al., 1994; Filvaroff, Stern et. al., 1990; Zhao, Sudol et. al., 1992; Zhao, Uyttendaele et. al., 1993). This increase occures within 5 minutes of Ca^{2+} addition and is blocked in the presence of the tyrosine kinase inhibitor genestein. This compound also interferes with Ca^{2+}-induced differentiation (Filvaroff, Stern et. al., 1990). Additionally, the stimulation in phosphorylation was not affected by the protein synthesis inhibitor cyclohexamide. These data suggest that tyrosine phosphorylation is a proximal event in Ca^{2+}-sensitive pathways leading to keratinocyte differentiation (Filvaroff, Stern et. al., 1990).

Tyrosine Phosphorylation by Receptor Tyrosine Kinases.

Since the 1950's it has been known that enzyme activity can be regulated by phosphorylation. Enzyme kinases covalently attach the gamma phosphate group from ATP to a serine, threonine, or tyrosine reside of the

target protein. This modification is subsequently reversed by the action of a phosphatase, which removes the phosphate group by hydrolysis.

Tyrosine phosphorylation comprises less than 5% of total cellular phosphorylation, yet it plays a vital role in signaling pathways of hormone action, cell growth and differentiation. Based on their location, protein tyrosine kinases (PTK's) are classified into two broad catagories: receptor tyrosine kinases (predominantly growth factor receptors) and cytoplasmic PTK's.

As their name suggests, receptor tyrosine kinases (RTK's) are activated by extracellular ligands such as growth factors and other cytokines (reviewed in van der Geer, Hunter et. al., 1994; Yarden and Ullrich, 1988). Among these growth factors are epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin and insulin growth factor (IGF), and cytokines such as colony stimulating factor 1 (CSF-1).

RTK's basic structure consists of a large extracellular ligand-binding domain, a single membrane-spanning segment, and an intracellular catalytic domain. Upon ligand binding the receptor dimerizes and transphosphorylates the C-terminal tail, thereby fully activating the kinase domain (reviewed in Heldin, 1995). This conformational change allows for the phosphorylation of cytoplasmic substrates which have associated with the receptor.

Many of the pathways activated by these signaling proteins converge on the small, membrane-bound guanine nucleotide binding protein Ras. Members of the Ras family act as molecular switches for many cellular processes leading to growth and differentiation (reviewed in Bollag and McCormick, 1991; Marshall, 1993; Pazin and Williams, 1992; Wittinghofer and Pai, 1991). Guanine nucleotide exchange factors (GEF's) such as Son-of-

sevenless (Sos) convert Ras to its GTP-bound active state. In this 'on' state, Ras can activate downstream signaling pathways such as the MAP kinase cascade. Indeed, studies using blocking antibodies and dominant negative forms of Ras have demonstrated the requirement of Ras for a multitude of cellular events including cell growth and differentiation (Cai, Szeberenyi et. al., 1990; Deshpande and Kung, 1987; Feig and Cooper, 1988; Hagag, Halegoua et. al., 1986; Mulcahy, Smith et. al., 1985; Szeberenyi, Cai et. al., 1990). Ras is then inactivated by hydrolysis of GTP to GDP. This weak intrinsic GTPase ability is increased up to 1000 fold in the presence of the GTPase activating protein, GAP (Bollag and McCormick, 1991; McCormick, Adari et. al., 1988; Trahey and McCormick, 1989 *and references therein*). Mutations in Ras which interfere with its interactions with GAP result in oncogenic forms of Ras.

Cytoplasmic PTK's

Unlike their transmembrane counterparts, cytoplasmic PTK's can be activated by a wide variety of signaling pathways. Among these are G-protein coupled receptors, ion influx, and even other tyrosine kinases (Brickell, 1992; Calautti, Missero et. al., 1995; Dikic, Tokiwa et. al., 1996; Lev, Moreno et. al., 1995; Zhao, Uyttendaele et. al., 1993 *and references therein*). For this reason they are thought to have a role in the amplification of the extracellular signal, rather than as a primary receptor of the signal.

The first and perhaps best studied of all tyrosine kinases is the cytoplasmic PTK Src kinase. v-Src was originally identified as the transforming protein of the avian rous sarcoma virus (RSV) (Erickson, Collett et. al., 1979; Hunter and Sefton, 1980; Jove and Hanafusa, 1987). Its cellular proto-oncogene, c-Src, has proved to be the prototype of cytoplasmic

PTK's. The study of this kinase has significantly advanced our understanding of the structure, regulation and substrates of signaling pathways involving tyrosine phosphorylation (reviewed in Brickell, 1992; Taylor and Shalloway, 1993).

Starting at the N-terminal of the Src gene product, the conserved regions of cytoplasmic PTK's include the Src homology 2 and 3 (SH2 and SH3) domains, the catalytic domain, and a short C-terminal tail. SH2 domains are a key component in tyrosine kinase cascades, as they bind phosphorylated tyrosine residues to form heteromeric signaling complexes. SH3 domains are involved in protein-protein interactions and have been shown to bind to proline-rich regions in the cytoskeleton and other signaling molecules. Homologs of these domains are found in a wide variety of other proteins including protein tyrosine phosphatases (PTP's), Ras GTPase activating protein (GAP), phospholipase C, adaptor proteins such as Shc, GRB2, and even myosin.

Overexpression or unregulated kinase activity of cytoplasmic PTK's can result in a plethora of pathological states. For example, the fusion protein resulting from the Philadelphia chromosome rearrangement has been identified as the cytoplasmic PTK Bcr-Abl. The enhanced activity of this kinase is responsible for the linked disease chronic myloid leukemia (CML) (McLaughlin, Chianese et. al., 1987). Similarly, the introduction of v-Src or the overexpression of c-Src results in unregulated cell growth, loss of differentiation, and gain of cell invasiveness when introduced into cultured cells (Behrens, Vakaet et. al., 1993; Brickell, 1992; Jove and Hanafusa, 1987).

There is also considerable redundancy in the ability of Src family members to phosphorylate various substrates. Transgenic studies by Soriano et. al. have demonstrate that Src is not required for general cell viability

(Soriano, Montgomery et. al., 1991). Intercrossing of $Src^{+/-}$ heterozygotes gave rise to live born homozygotes ($Src^{-/-}$). Histological examination of these Src deficient mice did not detect any gross abnormalities, with the exception of osteopetrosis; a condition associated with impaired osteoclast function. In subsequent transgenic studies, the Soriano group was similarly unable to detect an overt phenotype in mice deficient in other Src family members, including Fyn and Yes (Lowell and Soriano, 1996; Stein, Vogel et. al., 1994). The group attributes this lack of abnormalities to the functional overlap of these family members.

c-Src as a Downstream Effector of 7TM Receptors

c-Src and the adapter protein Shc have been the focus of recent attention as potential effectors of 7TM receptor signaling. Lefkowitz et. al. have recently characterized the activation of c-Src with effectors of the α-adrenergic receptor (α-AR) and the lysophosphatidic acid (LPA) receptor (Luttrell, Hawes et. al., 1996; van Biesen, Hawes et. al., 1995). Stimulation of these receptors has been shown to mediate the rapid activation of MAP kinase and ultimately result in mitogenesis (Dikic, Tokiwa et. al., 1996; Hawes, van Biesen et. al., 1995; Hordijk, Verlaan et. al., 1994; Howe and Marshall, 1993; van Biesen, Hawes et. al., 1995). By immunoprecipitation analysis, Lefkowitz et. al. have demonstrated that stimulation of the α-AR with epinephrine results in the the activation of Src and its association with the adaptor protein Shc (Luttrell, Hawes et. al., 1996). These events are both pertussis toxin sensitive, as would be expected with a pathway involving G_i. Similar results were also observed by overexpressing the βγ-subunit of G_i, consistent with a role of Shc in the α-AR to Src pathway. Additionally, overexpression of Csk, which inactivates Src, inhibited LPA-mediated

activation of MAP kinase. Thus, Shc and Src may have important roles in the activation of MAP kinase by 7TM receptors. The specific mechanism leading to phosphorylation of Shc and activation of c-Src are still unclear, however.

Shc Phosphorylation and its Consequences

The Shc gene codes for three protein products of approximately 46, 52, and 66 kDa (Pelicci, Lanfrancone et. al., 1992). The $p46^{Shc}$ and $p52^{Shc}$ isoforms are encoded by a single mRNA, and are derived from two translation start sites. While these two isoforms appear to be ubiquitously expressed, $p66^{Shc}$ is absent in some hematopoietic cells. All of the isoforms share common structural similarites including SH2 domains, the tyrosine phosphorylated collagen homology region (CH1) and the recently characterized phosphotyrosine binding (PTB) domain (Bonfini, Migliaccio et. al., 1996; Pelicci, Lanfrancone et. al., 1992; van der Geer and Pawson, 1995 *and references therein*). The $p66^{Shc}$ isoform contains an additional collagen homology 2 (CH2) domain of undetermined function. Both the SH2 domain and the PTB domain allow for Shc's interaction with important molecules involved in tyrosine kinase signaling cascades, such as the adapter protein Grb2 and the Ras guanine nucleotide exchange factor, Sos (Chen, Grall et. al., 1996; Pronk, McGlade et. al., 1993; Rozakis-Adcock, McGlade et. al., 1992).

Induction of Shc tyrosine phosphorylation is not limited to stimulation of 7TM receptors. Shc phosphorylation results from the activation of RTK's including EGFR, PDGFR, insulin receptor, and nerve growth factor receptor (NGFR) (Pelicci, Lanfrancone et. al., 1992; Pronk, McGlade et. al., 1993; Rozakis-Adcock, McGlade et. al., 1992); from the activation of cytokine receptors such as IL-2, IL-3 and GM-CSF (Burns, Karnitz

et. al., 1993; Damen, Liu et. al., 1993; Zhu, Suen et. al., 1994); and the activation of G-protein coupled receptors including thyrotropin releasing hormome (TRH) receptor, the endothelin receptor, the lysophosphatidic acid (LPA) receptor, and the α2-adrenergic receptor (α2-AR) (Cazaubon, Ramos-Morales et. al., 1994; Chen, Grall et. al., 1996; Luttrell, Hawes et. al., 1996; Ohmichi, Sawada et. al., 1994; van Biesen, Hawes et. al., 1995).

By virtue of the Shc-Sos interaction and data presented by Lefkowitz, Shc is implicated as having a role in p21/Ras activation. In cells stimulated with EGF, Shc becomes phosphorylated and physically associates with the EGF receptor (Blaikie, 1994; Margolis, 1992). Formation of this EGFR-Shc complex is believed to accomodate the translocation of Sos from the cytosol to the membrane, thereby allowing for its activation of membrane-bound Ras (Bonfini, Migliaccio et. al., 1996). Indeed, formation of this Shc-dependent heteromeric complex has been shown to promote Ras GTP-charging, thereby placing Ras in the "on" state (Pronk, Alida et. al., 1994).

As described above, activation of Ras has been shown to be required for various cellular events, including cell growth and differentiation. It is interesting to note that a role for Shc has been documented for both of these genetic programs. In PC12 cells, overexpression of Shc is sufficient to mimic NGF-induced neurite outgrowth, a classic marker of cell differentiation (Rozakis-Adcock, McGlade et. al., 1992). This effect was blocked by co-expression of dominant negative forms of Ras, again supporting a role for Shc as an upstream regulator of Ras. Shc overexpression has also been shown to be transforming in mouse fibroblasts, as monitored by tumor formation in nude mice (Pelicci, Lanfrancone et. al., 1992). Thus, Shc may effect signaling pathways leading to either proliferation or differentiation, depending on the cell type.

Negative Regulation of Ras By Tyrosine Phosphorylation.

The inactivation of Ras is also mediated by cellular events involving tyrosine phosphorylation. As described above, the intrinsic GTPase activity of Ras is increased up to 1000 fold in the presence of GAP (Bollag and McCormick, 1991; McCormick, Adari et. al., 1988; Trahey and McCormick, 1989 *and references therein*). Prior to stimulation, GAP is predominantly found in the cytosolic fraction of cellular lysates, physically separated from membrane-associated Ras (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991). Activation of tyrosine kinases such as the EGF and PDGF receptors and Src kinase results in the translocation of GAP to the membrane fraction (Neet and Hunter, 1995; Park and Jove, 1993). This translocation places GAP in proximity with Ras, thereby enhancing the rate of Ras-GTP hydrolysis. Pawson and co-workers have shown that GAP's translocation to the membrane is dependent upon the tyrosine binding SH2 domains of GAP (Marengere and Pawson, 1992; Moran, Polakis et. al., 1991).

Mutations to this SH2 domain also inhibit the interactions between GAP and another signaling protein implicated in Ras activity, GAP-associated p62. As its name suggests, p62 was first characterized by its presence in immunoprecipitations using anti-GAP antibodies. When anti-GAP immunocomplexes are incubated with anti-PY a highly phosphorylated protein of approximately 62 kDa is detected (Bouton, Kanner et. al., 1991; Ellis, Moran et. al., 1990). This phosphorylation is observed in response to a host of agonists and growth factors, including EGF, PDGF, Ca^{2+} and insulin (Ellis, Moran et. al., 1990; Hosomi, Shii et. al., 1994; Kaplan, Morrison et. al., 1990; Medema, De Vries-Smits et. al., 1995; Moran, Polakis et. al., 1991). An increase in the amount of tyrosine phosphorylated p62 associated with GAP

appears to be a ubiquitous response to these agonists, as it has been observed in tissues ranging from fibroblasts to lymphocytes.

The influence of the GAP-p62 complex on Ras activity is still unclear. There is increasing evidence that p62 may act as a membrane docking protein for GAP and other signaling molecules (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991; Neet and Hunter, 1995). GAP, for instance, is found predominantly in the membrane fraction when it is bound to p62 (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991). This translocation places GAP in close proximity to Ras, thereby enabling GAP to inactivate Ras. p62 may therefore be a indirect regulator of Ras. Further elucidation of p62's role in Ras signaling awaits cloning and characterization of the GAP-associated p62 gene and protein.

Phosphorylation of GAP-Associated p62 in Response to Elevation of Extracellular Ca^{2+}.

It appears that the increase in the amount of tyrosine phosphorylated p62 associated with GAP may also play a role in the Ca^{2+}-induced differentiation of keratinocytes. Early work by Filvaroff et al demonstrated that tyrosine phosphorylation is a required step in Ca^{2+}-induced keratinocyte differentiation. Furthermore, keratinocytes treated with high Ca^{2+} had increased tyrosine phosphorylation of a band of approximately 62 kilodaltons (kDa) when compared to their unstimulated counterparts (Filvaroff, Stern et. al., 1990). This protein has since been identified as the GAP-associated protein p62 (Filvaroff, Calautti et. al., 1992). As noted above, increases in the amount of tyrosine phosphorylated p62 associated with GAP have been documented in response to a host of agonists and growth factors (Ellis, Moran et. al., 1990; Hosomi, Shii et. al., 1994; Kaplan, Morrison et. al., 1990; Medema,

De Vries-Smits et. al., 1995; Moran, Polakis et. al., 1991).

In keratinocytes, however, an increase in the amount of tyrosine phosphorylated p62 associated with GAP has been reported to be a Ca^{2+}-specific event. Filvaroff and collegues have observed a robust phosphorylation of a 62kDa band in the GAP immunocomplex within 5 minutes of Ca^{2+} addition to cultured keratinocytes (Filvaroff, Calautti et. al., 1992; Filvaroff, Stern et. al., 1990). This increase was not observed when the cells were treated with EGF, phorbol esters or other growth factors, even though these agonists stimulated the tyrosine phosphorylation of other proteins such as PI_3 kinase and PLCγ, indicating that their specific tyrosine kinase pathways were still intact. The phosphorylation state of PI_3 kinase and PLCγ was not altered following Ca^{2+} stimulation, however. The authors therefore concluded that the increase in the amount of tyrosine phosphorylated p62 associated with GAP was an early and specific event in Ca^{2+}-induced keratinocyte differentiation (Filvaroff, Calautti et. al., 1992).

Stimulation of keratinocytes with extracellular Ca^{2+} is also accompanied by Ca^{2+} influx, Ca^{2+} release and an increase in $[Ca^{2+}]_i$. Filvaroff and collegues therefore attempted to distinguish between extracellular and intracellular calcium as a modulator of the amount of tyrosine phosphorylated p62 present with GAP. Signalling pathways sensitive to Ca^{2+} influx or release can be triggered with Ca^{2+} ionophores such as A23187 and X537A. Neither of these agents were able to stimulate the phosphorylation of the GAP-associated 62kDa band in keratinocytes (Filvaroff, Calautti et. al., 1994). Futhermore, conditioned media from keratinocytes stimulated with high Ca^{2+} was added to proliferating cells in the presence or absence of the Ca^{2+} chelator EGTA. This agent was able to block the Ca^{2+}-induced increase in the amount of tyrosine phosphorylated p62 associated with GAP, but was

titratable by molar excess of extracellular Ca^{2+}. These data demonstrate the reliance of the amount of phosphorylated p62 associated with GAP on extracellular Ca^{2+} levels, rather than through Ca^{2+} influx or a diffusible factor (Filvaroff, Calautti et. al., 1994).

Ca2+-stimulated tyrosine phosphorylation in fibroblasts.

The Ca^{2+}-stimulated increase in the amount of tyrosine phosphorylated p62 associated with GAP is not limited to keratinocytes. A14 and Rat1 fibroblasts cultured in low Ca^{2+} -containing media and then stimulated with 2 mM Ca^{2+} also show increased tyrosine phosphorylation as in keratinocytes. Under these conditions, Bos et. al. observed an increase in the tyrosine phosphorylation state of a doublet of approximately 62 and 65 kDa in anti-GAP immunoprecipitates from these two cell lines (Medema, De Vries-Smits et. al., 1995). One band of the doublet appeared to be specific for Ca^{2+}-induced tyrosine phosphorylation in the A14 cells.

In contrast to the differentiating response in keratinocytes, the stimulation of fibroblasts with external Ca^{2+} is associated with an increase in cell growth (Huang, Maher et. al., 1995). It may be that the increase in the amount of tyrosine phosphorylated p62 associated with GAP plays a role in the balance between cellular growth or differentiation, depending on the cell type and the presence or absence of specific signal transduction molecules. Again, further elucidation of this model awaits the cloning of the p62 cDNA.

Ca^{2+}-sensitive tyrosine kinases.

The PTK believed to be responsible for phosphorylating p62 *in vivo*, Src kinase, is also sensitive to changes in Ca^{2+} concentration. Zhao et. al. reported that Src kinase activity was elevated 5 to 6 fold in keratinocytes

following stimulation with a Ca^{2+}/A23187 mixture (Zhao, Sudol et. al., 1992). This activation was detectable within 30 minutes and was maintained for up to 6 hours. Conversely, a related Src-family member, c-Yes, is inactivated with this Ca^{2+}/ionophore mixture (Zhao, Uyttendaele et. al., 1993). The specific role of external Ca^{2+} in modulating these PTK's was not reported.

Src activity has been implicated in differentiation of other cell types as well. In the rat pheochromocytoma cell line PC12, treatment with nerve growth factor (NGF) induces terminal differentiation as monitored by neurite outgrowth. Microinjection of anti-src antibodies has been shown to inhibit this NGF-induced differentiation (Kremer, D'Arcangelo et. al., 1991). Furthermore, expression of the constitutively active form of the kinase, v-src, elicits neurite outgrowth in these cells (Thomas, Hayes et. al., 1991).

Another cytoplasmic PTK stimulated by changes in Ca^{2+}_i is the recently described Pyk2 kinase which is predominantly expressed in the brain and kidney (Lev, Moreno et. al., 1995). Pyk2 is activated by certain G-protein coupled receptors, phorbol esters, and the Ca^{2+} ionophore A23187. In PC12 cells bradykinin increases intracellular Ca^{2+} concentration by a pathway involving a G-protein coupled receptor and IP_3 release. Treatment of PC12 cells with this agonist results in a rapid (less than 1 minute) activation of Pyk2, as measured by autophosphorylation. Bradykinin induced Pyk2 activity when the Ca^{2+} chelator EGTA was present in the media. This data led the authors to conclude that Pyk2 activation is a direct response to elevation of Ca^{2+}_i, rather than through influx of this ion (Lev, Moreno et. al., 1995).

In summary, extracellular calcium plays a pivotal role in signaling pathways leading to growth and differentiation in a number of cell types.

Although the specific mechanisms through which the Ca^{2+} -dependent signals are transduced is unclear, it appears that a common event in these processes is tyrosine phosphorylation. Specifically, growth related proteins including c-Src and GAP-associated p62 have been reported to be tyrosine phosphorylated in response to elevation of extracellular calcium.

Filvaroff and co-workers have reported a similar increase in the amount of tyrosine phosphorylated p62 associated with GAP when keratinocytes are treated with polyvalent cations (Filvaroff, Calautti et. al., 1994). They observed that cations such as Mg^{2+} , Ni^{2+} and La^{3+} could mimic the effects of elevated Ca^{2+} on phosphorylation of the GAP-associated 62kDa band, whereas ionophores could not. These findings led them to conclude that the transducing molecule is a polyvalent cation receptor.

One possible candidate for such a receptor is the CaSR. Several reports have demonstrated that the CaSR is activated in response to polyvalent cations (Bai, Quinn et. al., 1996; Brown, Gamba et. al., 1993; Brown, Pollak et. al., 1995a; Brown, Gamba et. al., 1993; Emanuel, Adler et. al., 1996; Riccardi, Park et. al., 1995; Ruat, Molliver et. al., 1995; Ruat, Snowman et. al., 1996). In this study we address the potential role of the CaSR as a transducer of the Ca^{2+}_{o}-induced signal and test the influence of the CaSR on tyrosine phosphorylation.

Hypothesis to be tested

Changes in extracellular calcium concentration modify the behavior of many cell types, including parathyroid cells, kidney epithelial cells, neurons, keratinocytes, and breast epithelial cells. A seven-transmembrane domain receptor capable of sensing millimolar changes in extracellular calcium has

recently been cloned from parathyroid and kidney cDNA libraries, but little is known about the intracellular consequences of receptor activation in non-parathyroid cell types. While some specific signaling responses to changes in extracellular calcium have been documented in keratinocytes and fibroblasts, the precise relationship between these changes and the calcium-sensing mechanism present in these cells is unknown. For fibroblasts, the molecule responsible for sensing extracellular calcium has not been identified. Based on our work and others, we have formulated the following hypotheses: *1) Rat1 fibroblasts express functional receptors for sensing changes in extracellular calcium. 2) Activation of these receptors by extracellular calcium leads to activation of Src tyrosine kinase, increased tyrosine phosphorylation of Shc, and an increase in the amount of tyrosine phosphorylated p62 associated with GAP.*

In this thesis, we present experimental evidence supporting both hypotheses. We have demonstrated the presence of a CaSR in Rat1 cells by a combination of RT-PCR, Northern analysis and immunoblotting with a affinity-purified antibody raised against the extracellular region of the CaSR.

The role of CaSR as a mediator of tyrosine phosphorylation was addressed by stably transfecting the CaSR and the R796W inactivating mutant in Rat1 cells. The ability of these stable lines to respond to calcium or gadolinium with changes in tyrosine phosphorylation was then determined by immunoprecipitating proteins of interest and immunoblotting with anti-PY.

MATERIALS AND METHODS

Tissue Culture.

Rat-1 fibroblasts were routinely grown at 37° C in 5% CO_2/95% air in Dulbecco Modified Eagles Medium (DMEM, BioWhittaker) supplemented with 10% bovine calf serum (Hyclone) and gentamicin (10 μg/ml). New cultures were started from frozen stocks every 4-6 weeks. For experiments where calcium concentration was specified, Hanks F-12 media used and brought up to the indicated concentration with calcium chloride. The calcium concentration of F-12 is approximately 0.3 mM.

Primary human epidermal keratinocytes (HEK's) and primary human dermal fibroblasts (HDF's) were obtained commercially from Cascade Biologics, Inc. (Portland, OR). HEK's were cultured at 37° C in 5% CO_2/95% air in Medium 154 (Cascade Biologics, Inc.) supplemented with Human Keratinocyte Growth Supplement (HKGS) and gentamicin (10 μg/ml). The calcium concentration for this medium is approximately 0.2 mM. HDF's were cultured at 37° C in 5% CO_2/95% air in Medium 106 (Cascade Biologics, Inc) supplemented with Low Serum Growth Supplement (LSGS, Cascade Biologics, Inc) and gentamicin (10 μg/ml). The calcium concentration of this medium is approximately 1.2 mM.

Reagents.

Antibodies. The monoclonal antiphosphotyrosine antibody (anti-P-Y, 4G10) was a generous gift from Brian Druker (Oregon Health Sciences Univ.). Monoclonal anti-GAP antibodies and protein A/G agarose were obtained from Santa Cruz Biotechnology. Monoclonal anti-src antibodies, polyclonal anti-GAP and anti-Shc antisera were from UBI. The affinity purified

polyclonal anti-CaSR antibody was obtained commercially from Applied Biosystems (Golden, UT). The monoclonal anti-p62 (2C4) antibody was a generous gift from Richard Roth (Hosomi, Shii et. al., 1994; Ogawa, Hosomi et. al., 1994).

^{3}H-Thymidine Assays.

HEK's. Cells were split into parallel 12 well plates and allowed to grow to 50% confluence in their respective media. ^{3}H-Thymidine was added 4 hours prior to harvest. Cell agonists were added 18 hours prior to harvest and were present thoughout the 4 hour labeling period. Wells were then washed three times in 0.5 ml of 10% trichloroacetic acid (TCA) for 10 minutes. Cells were then lysed with 0.5 ml of 0.2 M NaOH. 0.2 ml of this mixture was added to a 7 ml scintilation vial and neutralized with 0.1 ml of 0.4 M HCl. 5 ml of scintillation fluor (Biosafe) was added, vials were mixed thoroughly and counted on a liquid scintillation counter (Beckman LSC6500). Statistical analysis was conducted on Statview software.

HDF's. ^{3}H-Thymidine assays were conducted on HDF's as described above with the following exceptions. HDF's were allowed to grow to approximately 80% confluence in their respective medium. The cells were then incubated in low-calcium, serum free media (Medium 154 + HKGS) for 36 hours prior to agonist stimulation. Incubation times with label and agonists were the same as described for the HEK.

Immunoprecipitations.

Cells were grown to confluence and treated as described in the appropriate figure legend. Cellular proteins were harvested by washing twice with cold PBS (10 mM $NaPO_4$, 150 mM NaCl), lysed on ice in M-TG buffer (1%

TX-100, 10% glycerol, 20 mM HEPES pH8.0, 2 mM Na_3VO_4, 150 mM NaCl, 1 mM NaF, 1 mM PMSF, and 1% aprotinin), and lysates were cleared by centrifugation at 6000g for 10 minutes. In general, 500 μg of protein were used per immunoprecipitation, as determined by protein assay (BioRad). Lysates were promptly incubated with antibodies for at least 4 hours at room temperature followed by incubation with protein A/G agarose for at least 1 hour. The immune complex was collected by centrifugation at 10,000g for 5 minutes. The pellet was washed extensively with M-TG buffer and prepared for SDS-PAGE by addition of 1/2 volume of Laemmli buffer 2x (4% SDS, 40% glycerol, 125 mM Tris pH 6.8, 2% β-mercaptoethanol, 0.1% bromophenol blue), and boiled for 3 minutes.

Plasma membrane preparation.

Crude plasma membranes were isolated from Rat1 cells stably transfected with pcDNA3 vector, CaSR or R796W using the protocol described by Bai et. al. (Bai, Quinn et. al., 1996). Confluent cells in 150mm dishes were washed twice with cold PBS and scraped in 1ml of homogenization buffer (50 mM Tris 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 1 mM PMSF) on ice. The cells were homogenized with 15 strokes of a 1.5 ml dounce homogenizer using the 'B' pestle. The nuclei were then cleared by centrifugation at 800g for 10 minutes. The supernatant was transfered to a new eppendorf tube and the membrane fraction was pelleted by centrifugation at 43,000 x g in a TLA 100.3 rotor for 1 hour. The resulting pellet was resolubilized in homogenization buffer with 1% Triton X-100 added.

Immunoblotting.

Proteins were separated on denaturing polyacrylamide gels and transferred to PVDF membrane (Immobilon P, Millipore) by electroblotting. Membranes were first incubated in blocking solution (3% BSA, 0.05% NaN_3) for 1 hour at room temperature followed by incubation with the primary antibody overnight at 4° C in TTBS (0.05% Tween 20, 20mM Tris pH 7.5, 150mM NaCl). Membranes were washed 3 times in TTBS, incubated with appropriate anti-immunoglobulin/horseradish peroxidase conjugates (Santa Cruz Biotech.) for at least 2 hours and washed extensively in TTBS. Bands were then visualized by chemiluminescence (DuPont NEN). Membranes could then be stripped with PVDF stripping solution (62.5mM Tris 6.8, 2% SDS, 100mM β-mercaptoethanol) for 30 minutes at room temperature for further immunoblotting. Where indicated, resulting autoradiograms from at least three independent experiments were scanned and densitometry analysis was performed by NIH Image software.

RNA isolation and Northern blot analysis.

Total RNA was extracted from 10 cm plates of Rat1 cells, size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels and transferred to nylon membrane (Nytran, Schleicher & Schuell, Keene NH). The membrane was hybridized using a 3.7kb XbaI/BamHI fragment from the full length rat striatal CaSR cDNA clone (kindly provided by S. Snyder) as a probe (Ruat, Molliver et. al., 1995). The fragment was labelled with [^{32}P]-dCTP (NEN) by random primer extension. Hybridization was conducted at 42° C in a 50% formamide hybridization solution. The membrane was then washed 2 times in 2X SSC at 42° C and 2 times in 2X SSC at 50° C. Hybridization was detected by PhosphorImager analysis (Molecular Dynamics), following a 16

hour exposure of the phosphorimage screen.

Isolation of CaSR fragment by RT PCR.

First strand cDNA for PCR analysis was generated from Rat1 cells using Superscript RT (Gibco-BRL). 5 µg total RNA and 0.5 µg of oligo dT were heated to 70° C for 10 minutes and placed on ice. The RT reaction consisted of the above plus First Strand buffer, 10 mM DTT, and 500uM dNTP's to a final volume of 24 µl. This mixture was heated to 42° C for 2 minutes then 1µl SuperScript RT was added and incubated for 50 minutes at 42° C. RT was inactivated by heating to 70° C for 15 minutes. 1ul of this reaction was PCR amplified in a 50 µl reaction containing Taq polymerase buffer, 500 µM dNTP's, 2 mM $MgCl_2$, 2.5 U of Taq polymerase (Gibco-BRL), and 100 pmoles of forward and reverse primers. The sequences of these oligomers, KR1 and KR2, corresponded to residues 2281 to 2305 and 3072 to 3096 respectively of the human CaSR (Garrett, Capuano et. al., 1995):

KR1: TTC CGC AAC ACA CCC ATT GTC AAG G

KR2: GGA TCC CGT GGA GCC TCC AAG GCT G

PCR was carried out for 30 cycles, each consisting of denaturation at 94° C for 1 minute, annealing at 55° C for 1 minute, extension at 72° C for 1 minute and with a final extension at 72° C for 5 minutes. The sample was electrophoresed on a 1% agarose gel and visualized by ethidium bromide flourescence. The expected 800 bp band was visualized and excised and DNA was purified by Wizard Prep (Promega). The fragment was subcloned into pGEM cloning vector using the pGEM-T kit (Promega). The resulting construct was introduced into ultracompetent JM109 bacterial cells and plated on LB/AMP containing IPTG/X-Gal. Plasmids from white colonies were isolated by standard techniques (Mantiatis) and sequenced by automated sequencer using

T7 primer at the Molecular, Microbiology and Immunology core facility at O.H.S.U. Sequence homology comparisons were made using Intelligenetics and MacVector software against existing databanks.

Generation of wild-type and mutant CaSR constructs.

pcDNA3-CaSR (CaSR): The full length 3.7kb CaSR clone was excised from pCIS:CaSR using XbaI-BamHI (Ruat, Molliver et. al., 1995). This was subcloned into pBluescript II (pBS II) for ease of manipulations. The full length 3.7kb CaSR from the pBS:CaSR construct was subcloned into the pcDNA3 mammalian expression vector using XbaI-XhoI.

pcDNA3-CaSR:R796W (R796W): A 1.2kb Sph I fragment from pBS:CaSR was subcloned into pGEM4Z. The R796W mutation was introduced into this construct by inverse PCR. The 50 µl PCR reaction was performed under the same conditions described above with the exception that the extensions were for 4 minutes and 20 ng of pGEM:1.2Sph DNA was used as a template. The sequence of the inverse oligomers J5 and J3 were as follows:

J5: TTG AAG GCA AAG AAG AAG CAG ATG G

J3: GTC CTG GAA GTT ACC CGA GAA CTT C

Primer J5 is 100% homologous with the published rat CaSR sequence. Primer J3 introduces the Family J mutation (R796W) and adds a diagnostic AvaI site. The expected 3.9kb product was purified by Wizard Prep. The linear DNA was phosphorylated with T4 kinase (Pharmacia), ligated together and introduced into ultracompetent XL-1 bacterial cells. Two positive colonies indentified by AvaI digests were grown up and resulting plasmids sequenced as noted above. The mutant 1.2bp Sph I fragment was then resubcloned into the pBS:CaSR contruct. Correct orientation was verified by AvaI digests. The

full length mutant CaSR (R796W) was then subcloned into the XbaI-XhoI sites of pcDNA3 as described above.

Transfection of cells.

Rat1 cells were seeded at $5x10^6$ cells per 100 mm plate or $3x10^4$ cells per well of a 6 well (35 mm) plate. The equivalent of 5μg/35 mm plate of purified DNA in serum-free medium was added to Lipofectin in serum-free medium and incubated at room temperature for 15-45 min. The cells were washed 3 times with serum-free DMEM and the DNA-lipofectin mix was then added. The cells were incubated at 37^0 C for 5 hrs, fed with medium containing 20% fetal calf serum (final serum concentration = 10%) and incubated 24-48 hrs. Cells were selected in media containing 700 μg/ml G418 (Bio-Whitaker) and incubated for another 72 hours. Cells were then seeded to allow for isolation of a single colony. Seven colonies were isolated from both the CaSR or R796W transfected plates. A single clone from each of the stable cell lines was selected based on their responsiveness to Ca^{2+}_o-stimulation, as monitored by increases in the amount of tyrosine phosphorylated p62 associated with GAP. Stable clones were cultured in 300 μg/ml G418 to maintain selection. New cultures were started from frozen stocks every 6-8 weeks.

***In vitro* kinase assays.**

Anti-src immunoprecipitations were performed as described above. Src kinase reactions were conducted in 50 μl of kinase buffer (30 mM Tris 7.5, 5 mM $MgCl_2$, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.4% aprotinin) containing 5μCi [γ-^{32}P] ATP (6000 Ci/mmole) and incubated at 20^0C for 10 minutes. Reactions were stopped with Laemmli buffer 2X, boiled for 2 minutes, and proteins were separated on denaturing polyacrylamide gels.

Gels were dried for 1 hour at 80^0 C and labelled proteins were visualized and quantitated by PhosphorImager.

Growth curves.

Parallel plates of Rat1 cells stably transfected with either vector alone (control), pcDNA3-CaSR or pcDNA3-CaSR:R796W were seeded at $1x10^4$ cells/well in 6 well plates and allowed to grow in DMEM supplemented with 0.5% bovine calf serum and gentamicin (10 μg/ml). Triplicate wells of each cell type were counted at the indicated time by washing with serum-free DMEM, trypsinized and thoroughly resuspended in DMEM for a final volume of 1ml. 0.5 ml of this was added to 9.5 mls of Isotone and cells were counted via Coulter Counter.

RESULTS

I. Effect of Agonists of the Calcium Sensing Receptor on Proliferation of Human Fibroblasts and Keratinocytes.

A. Thymidine Incorporation

Extracellular calcium (Ca^{2+}_o) appears to play a key role in pathways leading to cell growth and differentiation. Human fibroblasts, for example, are quiescent if cultured in low (0.1mM) calcium-containing medium, but replicate freely in 1mM calcium (Huang, Maher et. al., 1995; Morgan, Yang et. al., 1991). The addition of high calcium to cultured human keratinocytes, conversely, triggers terminal differentiation (Hennings, Holbrook et. al., 1983; Hennings, Michael et. al., 1980). It is our hypothesis that the molecule responsible for transducing the extracellular calcium-stimulated signal is the recently described calcium-sensing receptor (CaSR).

As an initial test of this hypothesis, we monitored the effect of agonists of the CaSR on ^{3}H-thymidine incorporation in human fibroblasts and keratinocytes. Primary human dermal fibroblast (HDF) cells were therefore grown in serum-free media under low Ca^{2+}_o conditions (0.2 mM) for 36 hours, the last 4 in the presence of ^{3}H-thymidine. When cells were exposed to varying concentrations of extracellular calcium for 18 hours prior to ^{3}H-thymidine addition (figure 1, panel A), a significant proliferative response was observed at a threshold [Ca^{2+}_o] between 1.2 mM and 1.6 mM (1.8-fold over basal), a 23-fold increase was observed in 1.8 mM [Ca^{2+}_o]. Stimulating the cells with magnesium (panel B) also resulted in an increase in ^{3}H-thymidine incorporation at the concentrations tested (5-20 mM). Both of these agents are capable of stimulating the CaSR within the dosages used here (Ruat, Snowman et. al., 1996). Thus, activators of the CaSR are capable of inducing increased ^{3}H-thymidine incorporation in HDF cells.

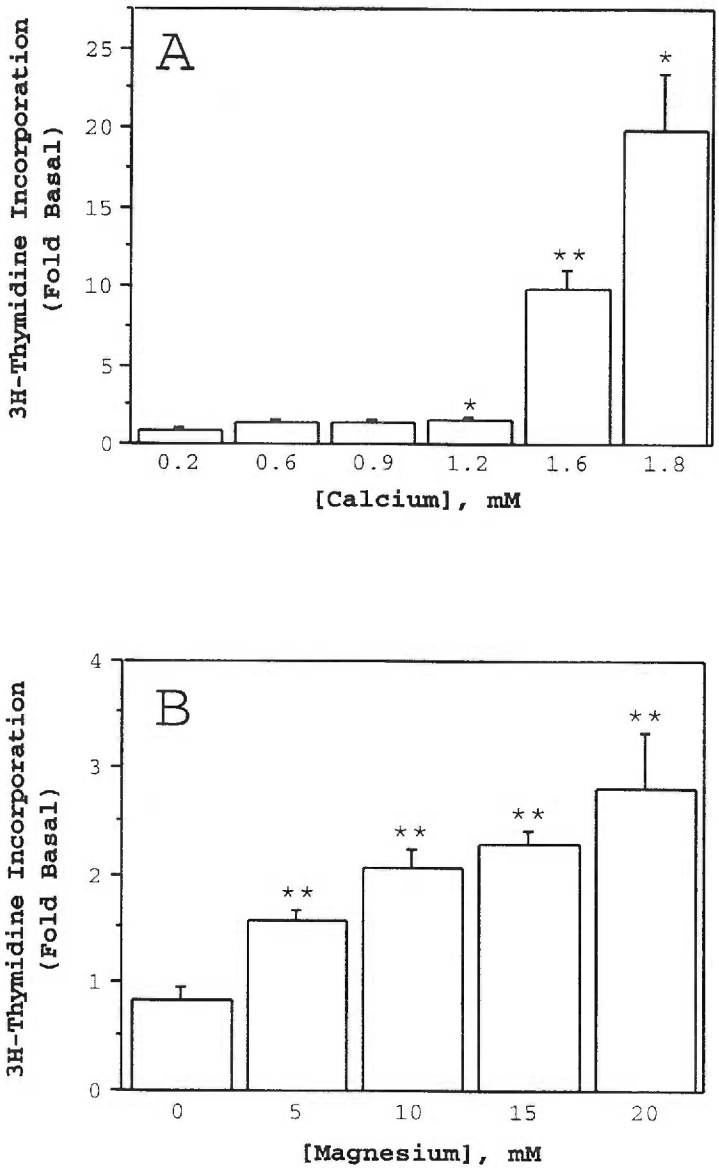

Figure 1. Human primary fibroblasts respond proliferatively to agonists of the CaSR. Cells were grown to 50% confluency then serum-starved in low calcium-containing media (0.2 mM) as described in *Materials and Methods*. A) Cells were stimulated with increasing amounts of extracellular calcium concentration (0.2-1.8 mM) for 18 hours and harvested for determination of ^{3}H-thymidine incorporation. B) Cells were stimulated with increasing amounts of magnesium for 18 hours and harvested for determination of ^{3}H-thymidine incorporation. Results are mean +/- S.D., n=4. One asterisk (*) denotes $p < 0.05$, two asterisks (**) denote $p < 0.01$ compared to basal levels at lowest concentration tested.

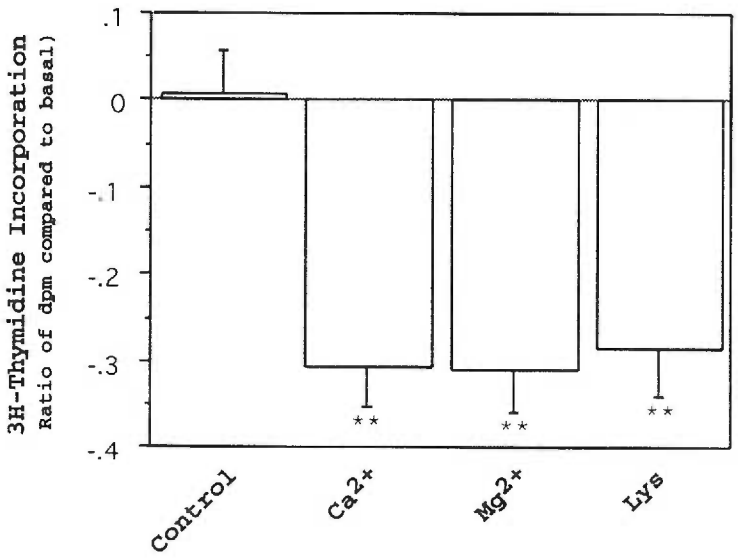

Figure 2. Agonists of the CaSR inhibit growth in human epidermal keratinocytes. Cells were grown to 50% confluency and labelled with ^{3}H-thymidine as described in *Materials and Methods*. Cells were stimulated with 1.8 mM calcium (Ca^{2+}), 10 mM magnesium (Mg^{2+}) or 10 nM polylysine (Lys) for 18 hours and harvested for determination of ^{3}H-thymidine incorporation. Results are mean +/- S.D., n=4. Asterisks (**) denote $p < 0.01$ compared to untreated control cells.

The effects of CaSR agonists were also tested on primary human epidermal keratinocyte (HEK) cells. Cells were exposed to extracellular calcium (1.8 mM), magnesium (10 mM) or the polyvalent cation, polylysine (100 nM) for 18 hours prior to the addition of ^{3}H-thymidine. Incorporation was measured after a 4 hour labelling period. As seen in figure 2, an inhibition of ^{3}H-thymidine incorporation was observed following treatment with each of these CaSR agonists. Stimulation of keratinocytes with agonists of the CaSR, therefore, is sufficient to inhibit ^{3}H-thymidine incorporation in HEK cells. Taken together, these results suggest the possibility that activation of the CaSR is capable of inducing proliferation or growth arrest, depending on the cell type.

To further address the possiblility that the CaSR might be a transducer of these extracellular signals, specifically those leading to proliferation in fibroblasts, we focused our attention on signaling pathways which are implicated in the proliferative response, namely those involving tyrosine phosphorylation.

B. $Ca^{2+}{}_o$-induced changes in cellular protein tyrosine phosphorylation.

Changes in the tyrosine phosphorylation state of many cellular proteins is seen in response to proliferative signals. Furthermore, changes in tyrosine phosphorylation of specifice proteins have been observed in keratinocytes exposed to elevated $Ca^{2+}{}_o$. To determine whether tyrosine phosphorylation in Rat1 fibroblasts could be induced by changes in $Ca^{2+}{}_o$, we examined the effect of elevating $Ca^{2+}{}_o$ on total tyrosine phosphorylation. Lysate from Rat1 cells cultured in low $[Ca^{2+}]_o$ (300uM) for 4 hours and then stimulated for 10 minutes with either 1.8mM Ca^{2+}, A23187, or both were subjected to SDS-PAGE and immunoblotted with anti-PY. A23187 is an

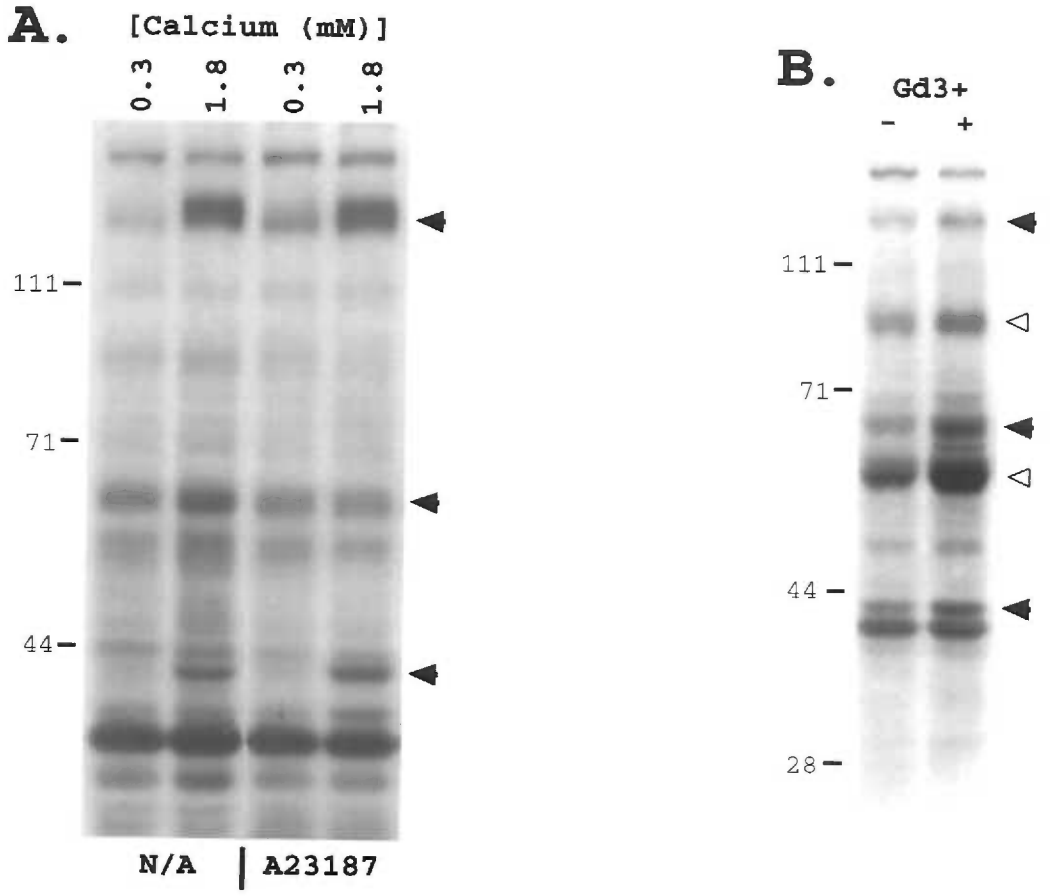

Figure 3. Calcium and gadolinium-induced tyrosine phosphorylation in Rat1 cells. Rat1 fibroblasts were cultured in low calcium containing media (300 μM) for 4 hours and then (A) stimulated with either calcium (1.8 mM), A23187 (1 μM), or both or (B) stimulated with 250 μM gadolinium (Gd^{3+}). Resulting lysates were resolved by SDS-PAGE and immunoblotted with anti-PY. Molecular weights in kilodaltons (kDa) are shown on the left.

ionophore which has the ability to release Ca^{2+} from intracellular stores in addition to promoting influx of the ion. This ionophore would then be expected to duplicate results arising from a change in intracellular calcium (Ca^{2+}_i) levels regardless of $[Ca^{2+}]_o$.

Elevation of $[Ca^{2+}]_o$ resulted in an increase in apparent tyrosine phosphorylation of proteins of approximately 41 kDa, 63 kDa, and a doublet of 135 kDa (figure 3A, arrowheads). Treatment with A23187 in the low calcium medium resulted in a slight increase in phosphorylation of the 135 kDa protein, but did not alter the observed intensity of the 41 kDa or 63 kDa bands. Stimulation with A23187 in the presence of 1.8 mM Ca^{2+} produced results similar to treatment with 1.8 mM Ca^{2+} alone, with the exception that the 63 kDa band lacked the apparent increase in phosphorylation. Thus ionophore in the presence of 300 uM Ca^{2+} could not mimic the observed effects of elevating $[Ca^{2+}]_o$ on the 41 kDa and 63 kDa proteins. This implies that elevation of Ca^{2+}_i alone is insufficient to induce tyrosine phosphorylation of the 41 and 63 kDa proteins, consistent with a receptor-mediated event.

C. Tyrosine phosphorylation in Rat1 cells following treatment with gadolinium.

The inability of A23187 to mimic the observed Ca^{2+}_o-induced changes in tyrosine phosphorylation argues against a mechanism based solely on increased levels of intracellular calcium. Filvaroff and co-workers suggest that the event triggering tyrosine phosphorylation in keratinocytes is the activation of a polyvalent cation receptor (Filvaroff, Calautti et. al., 1994). We hypothesize that this receptor is the CaSR, and that the CaSR is also expressed in Rat1 fibroblasts where it transduces Ca^{2+}_o-stimulated signals.

If the CaSR is indeed a mediator of Ca^{2+}_o-induced tyrosine

phosphorylation in Rat1 cells, then agonists of the CaSR would be expected to mimic the elevation in Ca^{2+}_o. To address this, we tested the ability of extracellular gadolinium (Gd^{3+}) to influence tyrosine phosphorylation in these cells. Gd^{3+} is a potent and specific agonist of the CaSR, with an EC_{50} of 60 μM and does not influx through ion channels (Brown, Pollak et. al., 1995b; Brown, Gamba et. al., 1993; Canzoniero, Taglialatela et. al., 1993; Racke, Dubyak et. al., 1991; Shoback and Chen, 1991). Rat1 cells were incubated in low Ca^{2+}-containing medium for four hours and treated with Gd^{3+} (250 μM) for 10 minutes. Lysates were subjected to SDS-PAGE and immunoblotted with anti-PY.

Figure 3B demonstrates that treatment with Gd^{3+} was capable of inducing tyrosine phosphorylation in total cell lysates from Rat1 cells. As was the case with calcium stimulation, treatment with Gd^{3+} resulted in an increase in the apparent phosphorylation of proteins corresponding to 41 kDa, 63 kDa, and 135 kDa (figure 3B, arrowheads). In addition to these, two proteins with approximate molecular weights of 55 kDa and 90 kDa were also observed to exhibit increased phosphorylation in response to Gd^{3+}-stimulation (figure 3B, open arrows). Thus, known agonists of the CaSR, in addition to Ca^{2+}_o, are also able to modulate tyrosine phosphorylation in Rat1 cells.

D. Effects of Ca^{2+}_o on the amount of phosphorylated p62 detected in GAP immunocomplexes.

One tyrosine phosphorylated protein which is thought to be sensitive to elevations in Ca^{2+}_o is the GAP-associated protein p62. The amount of tyrosine phosphorylated p62 present in anti-GAP immunocomplexes has been shown to increase in response to Ca^{2+}-stimulation in keratinocytes and

fibroblasts (Filvaroff, Calautti et. al., 1992; Filvaroff, Calautti et. al., 1994; Medema, De Vries-Smits et. al., 1995). Results depicted in figure 3 suggest that a protein of similar molecular weight exhibited increased tyrosine phosphorylation in our system. We therefore tested the effect of Ca^{2+}-stimulation on the amount of tyrosine phosphorylated p62 associated with GAP in Rat1 fibroblasts.

Rat1 cells cultured in low $[Ca^{2+}]_o$ (0.3mM) were exposed to 1.8mM calcium for 10 minutes and harvested. Resulting lysates were analyzed for phosphorylation of the 62 kDa GAP-associated band by immunoprecipitating with anti-GAP antibodies and immunoblotting with anti-phosphotyrosine antibodies (anti-PY). As seen in figure 4, the addition of calcium to these cells results in an increase in the apparent phosphorylation of a doublet of approximately 63kD, the approximate size for p62 (Bouton, Kanner et. al., 1991; Medema, De Vries-Smits et. al., 1995; Neet and Hunter, 1995). As a positive control, Rat1 cells were also stimulated with EGF or the phorbol ester TPA for 10 minutes. Calcium-induced phosphorylation of the 62 kDa GAP-associated band was similar in intensity to that observed in cells treated with TPA (lane 7), but less than that in cells stimulated with EGF (lane 8). The increase in the amount of tyrosine phosphorylated p62 associated with GAP following calcium stimulation was also decreased in the presence of the tyrosine kinase inhibitor herbimycin A (lanes 5 and 6), implicating activation of a cytoplasmic tyrosine kinase.

If the observed increase in the amount of tyrosine phosphorylated p62 associated with GAP resulted soly from an elevation of intracellular Ca^{2+} due to either influx or release, then agents that elevate intracellular Ca^{2+} independent of $Ca^{2+}{}_o$ would be expected to induce phosphorylation of the 62 kDa GAP-associated band. To address this possibility, Rat1 cells cultured in

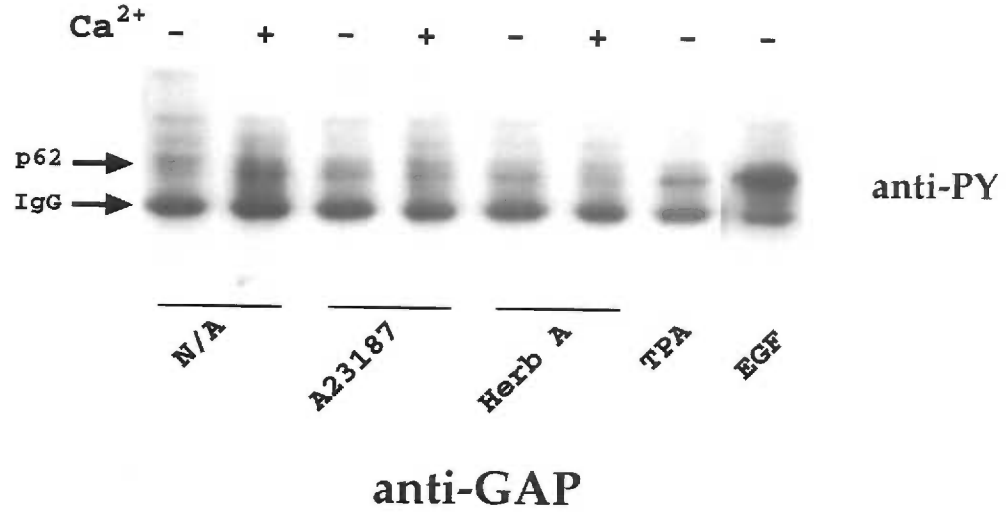

Figure 4. Calcium-induced GAP-associated p62 phosphorylation in Rat1 cells. Rat1 fibroblasts were cultured in low calcium containing media (300 μM, -) for 4 hours and then stimulated with calcium (1.8 mM, +), A23187 (1 μM), TPA (100 μM), or EGF (10 ng/ml) as indicated for 10 minutes and harvested in M-TG buffer. Cells were treated with 100 μg/ml Herbimycin A (Herb A) for 18 hours prior to calcium stimulation where indicated. Resulting lysates were immunoprecipitated with anti-GAP, subjected to SDS-PAGE and immunoblotted with anti-PY.

low Ca^{2+}_o were treated with the ionophore A23187 and prepared as above. A23187 treatment, either alone or in the presence of high Ca^{2+}_o, was not able to mimic the apparent increase in Ca^{2+}_o -induced phosphorylation of the 62 kDa band (figure 4, lanes 3 and 4).

II. Molecular Evidence of CaSR Expression in Rat1 cells.

Based on our preliminary results showing that known agonists of the CaSR could induce the same changes as elevating Ca^{2+}_o, we used a variety of techniques to determine whether the CaSR was expressed in Rat1 cells at both the mRNA and protein levels.

A. CaSR mRNA expression in Rat1 fibroblasts.

RT-PCR analysis. As an initial test for the presence of CaSR mRNA in Rat1 cells, we used RT-PCR reactions on total RNA from these cells. The oligonucleotides (KR1 and KR2, described in *Materials and Methods*) used in the reaction were directed against the transmembrane region of the receptor. This region of the CaSR is the most conserved between species, and shares little homology with other proteins in current databases.

Using these oligomers, a band of the predicted 800bp size was amplified and subcloned into the pGEM-T vector. There were no PCR products in the water controls, indicating that the observed 800bp band was not due to genomic contamination. The 800bp PCR product was sequenced on the MMI core facility automated sequencer using T7 as the primer. The resulting sequence gave a predicted amino acid sequence greater than 95% identical to the previously cloned rat CaSR (figure 5).

Northern blot analysis. The RT-PCR results were confirmed by

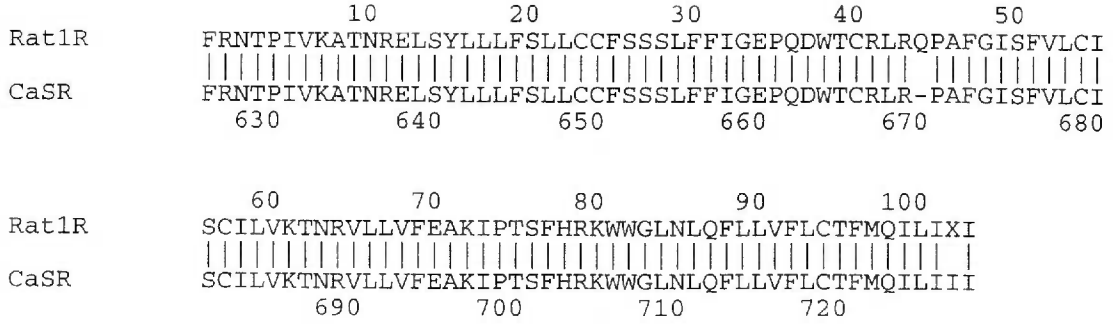

Figure 5. Sequence alignment between the published rat CaSR cDNA and the KR1-KR2 RT-PCR product. PCR amplication of the CaSR transcript using KR1 and KR2 oligonucleotides as described in *Material and Methods*. The expected 800bp product was subcloned and sequenced. The resulting amino acid sequence (Rat1R) is shown here aligned against the published rat CaSR sequence (CaSR), the numbers indicate the respective amino acid position.

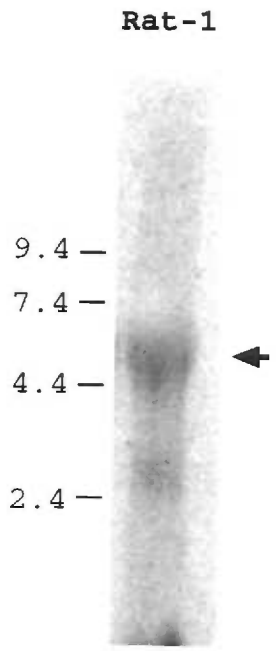

Figure 6. Expression of CaSR RNA in Rat1 cells. Total RNA from Rat1 cells was prepared and 20 μg was size fractionated as described in Materials and Methods. Northern hybridization was done with randomly primed 32P-labelled probe from the full length CaSR. Molecular weight markers in kilobases are shown on the left.

Northern hybridization analysis. Total RNA was purified from Rat1 cells, size fractionated by electrophoresis and subjected to Northern blot analysis using the full length rat CaSR as a probe (figure 6). A major 5.3 kb transcript was observed, consistent with that reported by Brown et. al. (Brown, Gamba et. al., 1993).

B. Western analysis of the CaSR in Rat1 cells

To verify the presence of the CaSR at the protein level, we collaborated with a commercial vendor in the production of a polyclonal antibody (anti-CaSR) raised against a 17 amino acid peptide sequence (KALAWHSSAYGPDQRAQ) in the extracellular domain of the rat CaSR. This sequence is identical to that successfully used to generate antibodies by Snyder and co-workers in their characterization of the rat CaSR (Ruat, Molliver et. al., 1995). The antibody was affinity purified against the CaSR-peptide used for immunization.

CaSR expression was assayed in partially purified plasma membrane proteins isolated from Rat1 cells. Immunoblot analysis using the affinity purified anti-CaSR antibody on this preparation revealed a band with an approximate molecular weight of 140kD (figure 7, panel A lane 1), consistent with the size reported by Snyder. The 140kD band was absent when the antibody was preincubated with the control peptide (figure 7, panel B lane 1).

III. Interactions Between The CaSR And Cellular Tyrosine Phosphorylation.

A. Western analysis of Rat1 cells stably transfected with the CaSR or the R796W mutant

The results in figures 3 through 6 demonstrate that tyrosine

phosphorylation is sensitive to Ca^{2+}_o in Rat1 fibroblasts. To test the influence of the CaSR on these events, we overexpressed the wild-type CaSR and a non-functional mutant (R796W) in Rat1 cells as described in *Materials and Methods* and observed the effect on the previously identified Ca^{2+}_o-sensitive responses. The R796W mutation has been shown to attenuate CaSR-sensitive signaling pathways both *in vivo* and *in vitro* (Bai, Quinn et. al., 1996; Pollak, Brown et. al., 1993). We predicted that those tyrosine phosphorylations dependent on CaSR function would be attenuated in the presence this mutant.

To demonstrate overexpression of these constructs, we conducted Western analysis using the anti-CaSR antibody on the Rat1 cells stably transfected with either the CaSR or R796W mutant. Since the interfering mutant only differs from the wild-type receptor in one residue in the cytoplasmic region, the existing anti-CaSR antibody would be expected to bind both the wild-type and R796W receptors. Partially purified membrane fractions from these cell lines were prepared by SDS-PAGE and immunoblotted with anti-CaSR as above. The 140kD band exhibited increased expression in the stable transfectants compared to cells transfected with vector alone (figure 7, panel A). This band was absent when the antibody was preincubated with the control peptide (figure 7, panel B).

B. Total tyrosine phosphorylation in cells transfected with wild-type and mutant CaSR

To test the effect of the CaSR on total tyrosine phosphorylation, we conducted Western analysis on total cell lysates from these stable cell lines. Rat1 cells stably transfected with either vector alone, the CaSR or the R796W interfering mutant were cultured in low Ca^{2+}-containing media (300 μM) for

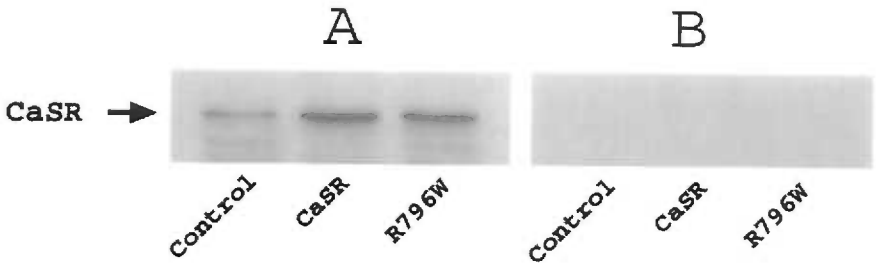

Figure 7. Western analysis of CaSR receptor in stably transfected Rat1 cells. Plasma membrane proteins from Control, CaSR, and R796W cell lines were isolated as described in *Material and Methods* and subjected to SDS-PAGE. Each lane contains 40 μg of protein lysate. Membrane was immunoblotted with (A) affinity purified anti-CaSR antibody or (B) with the same antibody preincubated with the specific peptide against which it was raised.

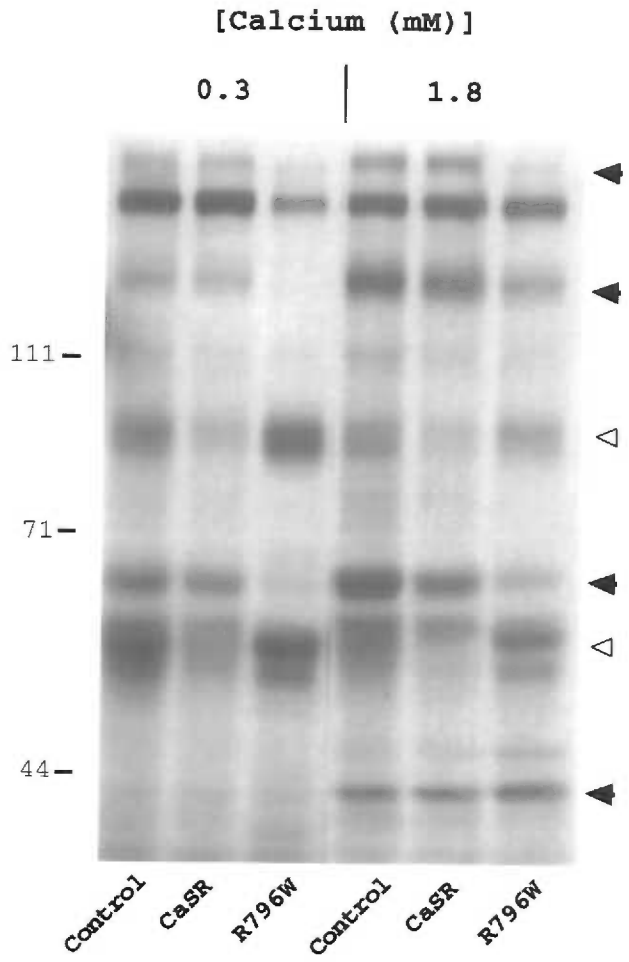

Figure 8. Effect of CaSR and interfering mutant on calcium-induced tyrosine phosphorylation. Rat1 cells stably transfected with vector alone (Control), the CaSR, or the R796W mutant were grown to confluence in normal DMEM then cultured in low calcium-containing media for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Lysates were resolved by SDS-PAGE and immunoblotted with anti-PY. Molecular weights in kilodaltons are shown on left.

4 hours and then exposed to 1.8 mM Ca^{2+}_o for 10 minutes. The resulting lysates were subjected to SDS-PAGE and immunoblotted with anti-PY.

As seen in figure 8, the addition of calcium to control cells and to cells overexpressing the CaSR resulted in an increase in the apparent phosphorylation of bands of approximately 41 kDa, 63 kDa, and 135 kDa (figure 8, arrowheads). In the R796W cells, however, the observed changes in total tyrosine phosphorylation in response to calcium stimulation varied widely. Bands corresponding to 63 kDa and 135 kDa, for instance, were attenuated in both untreated R796W cells and R796W cells stimulated with calcium. Conversely, bands of approximately 55 and 90 kDa appeared to have increased phosphorylation in low Ca^{2+} media compared to control cells. These 55 and 90 kDa bands actually decreased in intensity upon calcium addition (figure 8, open arrows). Thus the effect of the R796W mutant on CaSR function appears to be complex, with varying enhancement or inhibition of tyrosine phosphorylation depending on the particular protein.

C. Effect of elevating Ca^{2+}_o on p62 phosphorylation in the stable cell lines.

To assess the influence of the CaSR on Ca^{2+}-stimulated increase in the amount of tyrosine phosphorylated p62 present in the GAP immunocomplex, we treated the stable cell lines with 1.8 mM Ca^{2+} and immunoprecipitated the resulting lysates with anti-GAP antibodies. Immunoblotting with anti-PY revealed a Ca^{2+} -dependent increase ($p < .05$) in the amount of detected tyrosine phosphorylated p62 found in the GAP immunocomplex in the vector control and CaSR overexpressing cells (figure 9, arrow). The increase in phosphorylation was significantly diminished ($p < .05$) in the R796W cell line compared to the Ca^{2+}-stimulated control cells. Similar results were obtained (ie. increases with the CaSR and decreases with R796W cells) in 6 out

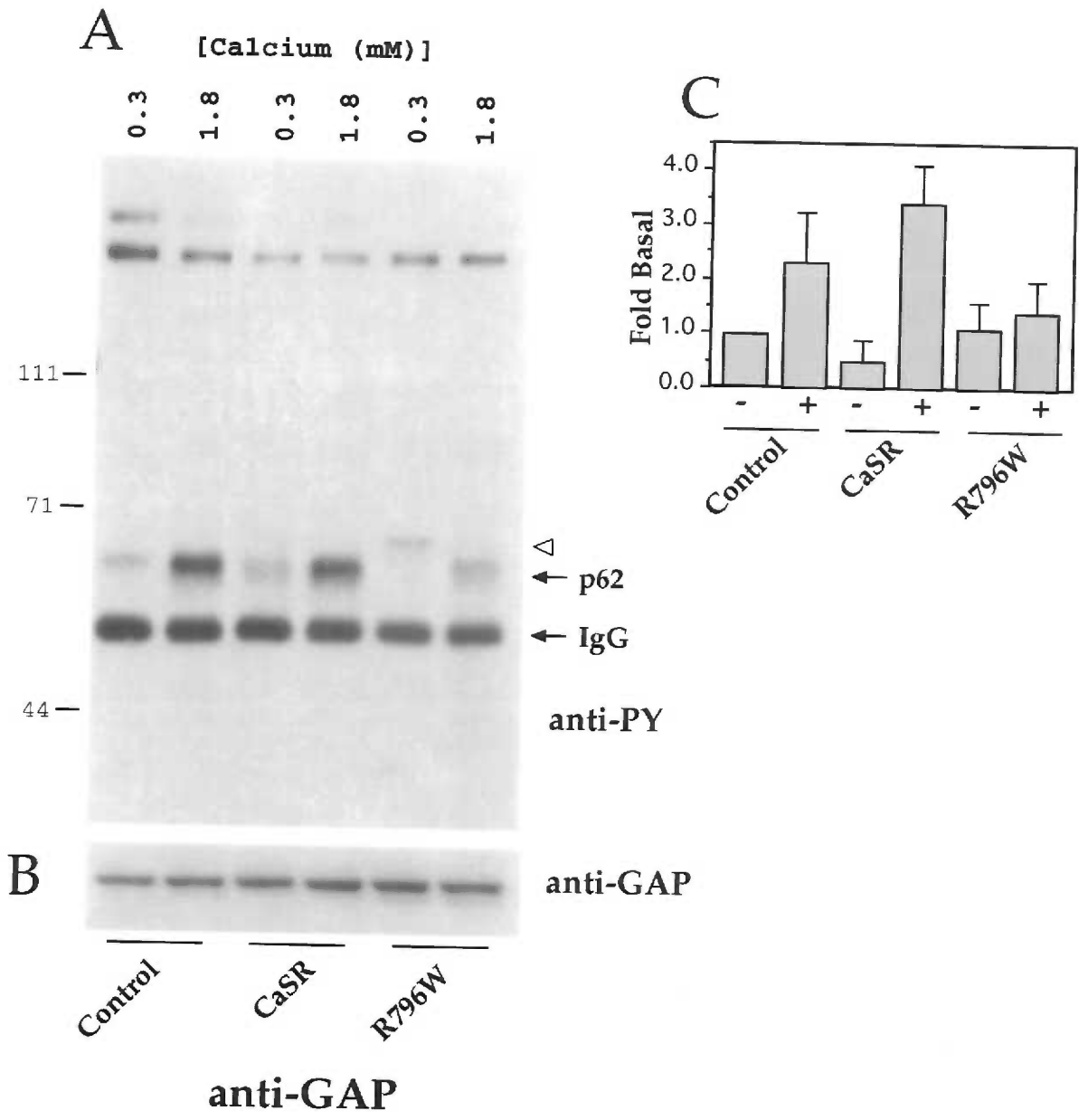

Figure 9. Effect of CaSR and interfering mutant on calcium-induced GAP-associated p62 phosphorylation. Rat1 cells stably transfected with vector alone (Control), the CaSR, or the R796W mutant were grown to confluence in normal DMEM then cultured in low calcium-containing media for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Resulting lysates were immunoprecipitated with anti-GAP, subjected to SDS-PAGE and immunoblotted with (A) anti-PY or (B) anti-GAP. Molecular weights in kDa are shown on the left. (C) GAP-associated p62 phosphorylation was determined by scanning densitometry. Data are presented as fold increase over nonstimulated controls and represent the means +/- S.D. for three independent experiments.

of 7 clonal isolates tested, indicating that these results were not an artifact of transfection. The lower panel (figure 9, panel B) demonstrates that relative levels of GAP protein precipitated were similar under these conditions. Band intensity of the 62 kDa protein for each of the samples, as measured by scanning densitometry and normalized to nonstimulated controls, is depicted in the histogram in panel C.

An additional GAP-associated protein of approximately 68 kDa observed in the untreated mutant cell line (figure 9A, open arrow) was reproducibly seen in 5 out of 7 colonies tested. The significance of this protein is unknown. The apparent changes in phosphorylation of the larger 160 kDa and 190 kDa proteins are believed to be artifactual, as their presence varied widely between colonies and individual experiments.

A single clone from each of the stable cell lines was selected at this point based on their responsiveness to Ca^{2+}_o-stimulation, as monitored by phosphorylation of GAP-associated p62. The clones were then used to further characterize the role of the CaSR as a transducer of the Ca^{2+}_o-sensitive signal.

D. Effect of calcium addition and the CaSR on the GAP-p62 complex.

GAP-associated p62 was originally identified as a tyrosine phosphorylated protein found in anti-GAP immunoprecipitates following stimulation by either PDGF, EGF, or insulin. The first reported cloning of p62 engendered confusion, as that cDNA actually encoded Sam68, and not GAP-associated p62 (Neet and Hunter, 1995). In human fibroblasts, Medema et. al. have also reported a novel GAP-associated tyrosine phosphorylated protein of approximately 65kD that was sensitive to changes in Ca^{2+}_o but not to insulin treatment (Medema, De Vries-Smits et. al., 1995). This p65 protein did not comigrate with the p62 that is tyrosine phosphorylated in response to insulin

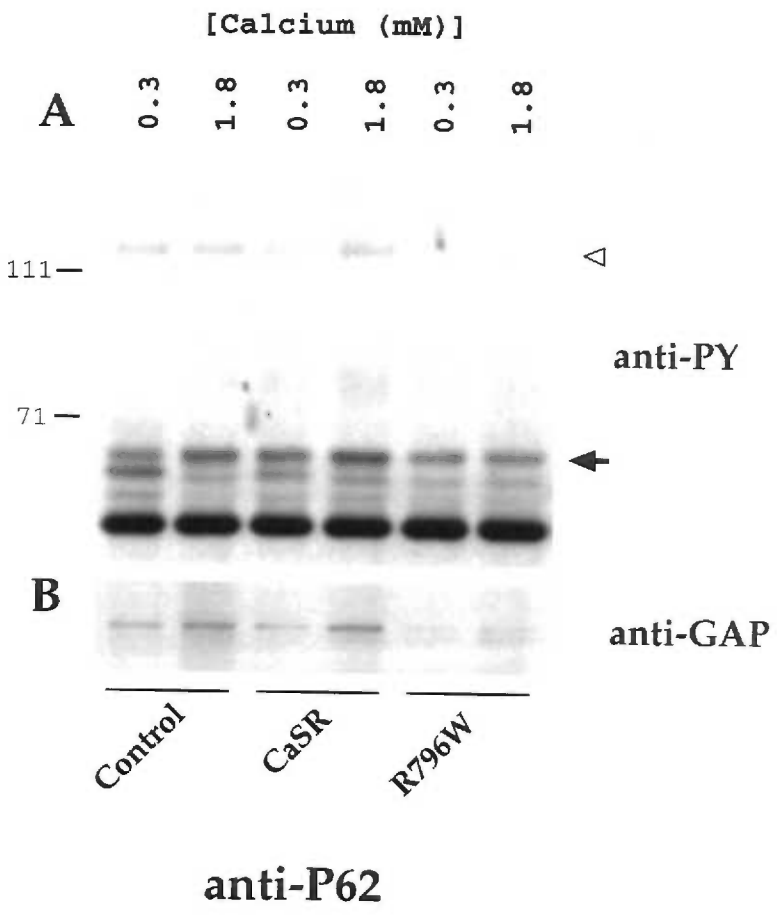

Figure 10. Effect of CaSR and interfering mutant on calcium-induced p62 phosphorylation and GAP association. Rat1 cells stably transfected with vector alone (Control), the CaSR, or the R796W mutant were grown to confluence in normal DMEM then cultured in low calcium-containing media for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Resulting lysates were immunoprecipitated with anti-p62 (2C4), subjected to SDS-PAGE and immunoblotted with (A) anti-PY. (B) The same blot was stripped and immunoblotted with anti-GAP. Molecular weights in kDa are shown on left.

addition.

To test whether the GAP-associated protein seen in our study is indeed the GAP-associated p62 (or p65), we obtained a monoclonal antibody which recognizes only the GAP-associated p62 (Hosomi, Shii et. al., 1994; Medema, De Vries-Smits et. al., 1995; Neet and Hunter, 1995). This antibody (2C4) immunoprecipitates p62, but does not detect the protein on Western blots. Lysates from the stable Rat1 cell lines treated with either 0.3 mM or 1.8 mM Ca^{2+} were immunoprecipitated with the anti-p62 antibody (2C4). Immunoblot analysis using anti-PY showed a protein of 62 kDa that was constutively phosphorylated regardless of $[Ca^{2+}]_o$ (figure 10A, arrow).

An additional protein of approximately 120 kD reacting to anti-PY was present in anti-p62 immunoprecipitates from the wild type and CaSR overexpressing cells, but was not observed in the R796W mutant cells (figure 10A, open arrow). Immunoblot analysis with anti-GAP antibodies identified this protein as p120/GAP (figure 10, panel B). The amount of GAP present in the p62 immunocomplex was observed to be increased upon exposure to high calcium in both the wild-type and CaSR overexpressors. In the R796W cells, however, p120/GAP protein was not detected in either the untreated or Ca^{2+}-stimulated cells. Similar results regarding the phosphorylation state of the 62 kDa protein and the absence of GAP in the R796W cell line were obtained in three replicate experiments.

These data suggest that formation of the GAP-p62 complex is sensitive to changes in extracellular calcium and that a functional CaSR may be required for the association of GAP with p62 in response to elevations in $[Ca^{2+}]_o$. The absence of GAP protein in these p62 immunoprecipitations when the R796W mutant is overexpressed also indicates that tyrosine phosphorylation of p62 is not sufficient for formation of the GAP-p62

complex.

F. Interactions between the CaSR and Src kinase

c-Src is known to show increased kinase activity in response to elevated Ca^{2+}_o in keratinocytes (Zhao, Sudol et. al., 1992). The Ca^{2+}_o-dependent changes in tyrosine phosphorylation seen in figures 3, 4, 8 and 9 might reflect a Ca^{2+}_o -sensitive activation of c-Src in Rat1 cells. This hypothesis implies that agonists of the CaSR should also activate Src kinase. Similarly, the presence of an interfering mutant of the CaSR should attenuate this response.

To test this hypothesis, we measured Gd^{3+}-stimulated Src activity in the stable cell lines, as measured by c-Src autophosphorylation. Parallel plates of Rat1 cells stably transfected with either vector, CaSR or R796W mutant were cultured in low Ca^{2+}-containing media for 4 hours. Cells were then stimulated with 250uM Gd^{3+} for 10 minutes and harvested. Resulting lysates were immunoprecipitated with anti-Src antibody and incubated with [^{32}P]-γATP as described in *Material and Methods*. As shown in figure 11, Gd^{3+} treatment produced similar 2.5 fold increases in c-Src autophosphorylation in both the vector control and CaSR overexpressing cells, suggesting that endogenous levels of the CaSR are probably sufficient for a maximal Src response to the dose of Gd^{3+} tested. Furthermore, the ability of Gd^{3+} to stimulate Src activity was significantly inhibited in cells transfected with the interfering mutant when compared to the increase observed for the control cells. The amounts of Src protein were similar in each of the transfected cell lines (figure 11, inset). These data suggest that this non-functional mutant of the CaSR can block activation of Src kinase by Gd^{3+}, further strengthening the hypothesis that CaSR activation leads to induction of Src kinase activity.

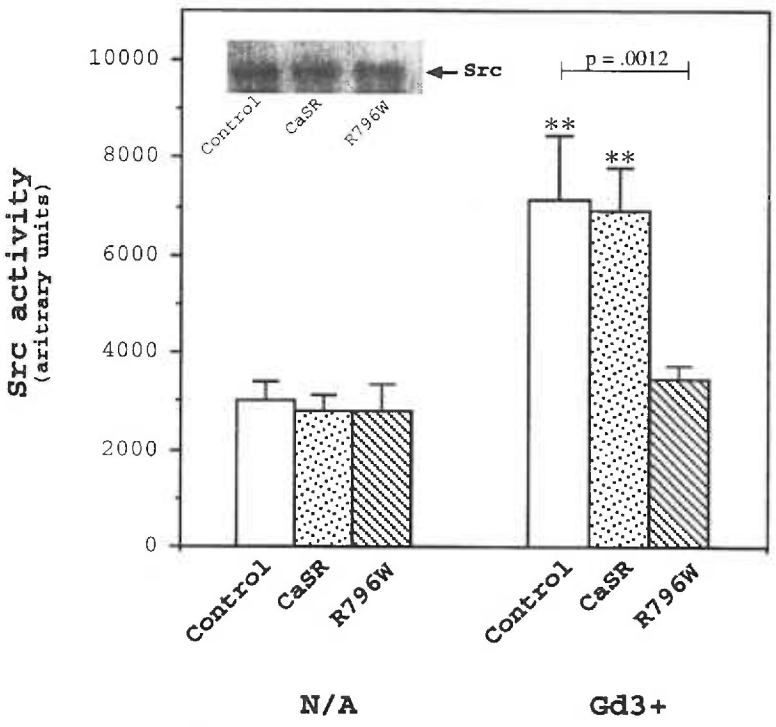

Figure 11. Effects of CaSR and R796W mutant on Gd^{3+}-stimulated Src activity. Rat1 cells stably transfected with vector alone (Control), the CaSR, or the R796W mutant were grown to confluence in normal DMEM (1.8 mM Ca^{2+}) then cultured in low (0.3 mM) calcium-containing media for 4 hours. Parallel plates were then treated with 250 μM Gd^{3+} for 10 minutes and harvested in M-TG buffer. Resulting lysates were immunoprecipitated with anti-Src antibodies and kinase activity was measured by autophosphorylation in the presence of [^{32}P] γ-ATP. Relative activity was quantified by PhosphorImager analysis, and is expressed as the mean +/- SD, n=4. To show relative protein levels, lysates were both immunoprecipitated and immunoblotted with anti-Src (inset). Asterisks (**) denote $p < 0.01$ compared to untreated controls.

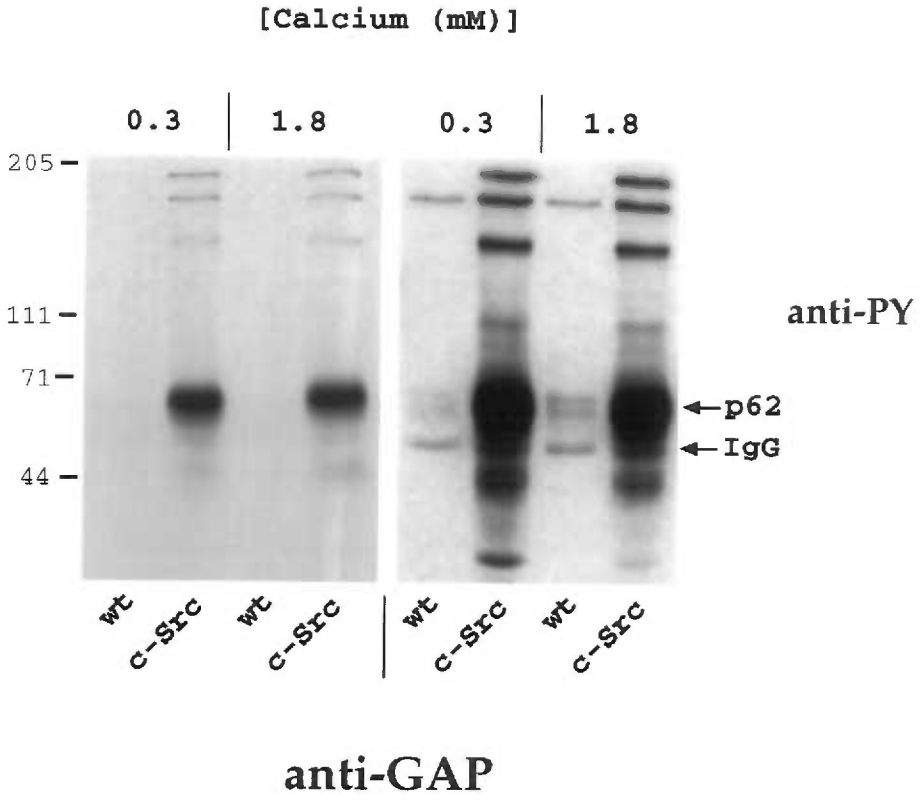

Figure 12. Effect of c-Src overexpression on calcium-induced GAP-associated p62 phosphorylation. Rat1 cells and Rat1's stably transfected with Src kinase were grown to confluence in normal DMEM then cultured in low calcium-containing media (0.3 mM) for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Resulting lysates were immunoprecipitated with anti-GAP, subjected to SDS-PAGE and immunoblotted with anti-PY. The left panel is a 20 second exposure, the right for 2 minutes. Molecular weight markers in kilodaltons are shown on the left.

To further investigate the possibility that activation of c-Src could contribute to the observed increase in phosphorylation of GAP-associated p62, we overexpressed c-Src in Rat1 fibroblasts and observed the effect on Ca^{2+}_o - induced GAP-associated p62 phosphorylation. Rat1 cells overexpressing c-Src were grown in 0.3 mM Ca^{2+} for 4 hours then exposed to 1.8 mM Ca^{2+}, harvested and immunoprecipitated with anti-GAP. Immunoblotting with anti-PY revealed intense tyrosine phosphorylation of the 62 kDa band, even in low Ca^{2+} conditions (figure 12, left panel). The relative amount of tyrosine phosphorylated p62 associated with GAP in these cells is dramatically greater than that in an equivalent amount of lysate from the wild-type cells (figure 12, right panel). Overexpression of c-Src protein, therefore, can overcome the normal sensitivity of the kinase to changes in extracellular calcium. Similar results were obtained in cells expressing the constitutively active form of Src, v-Src. In v-Src transformed cells we found the 62 kDa band to be highly phosphorylated even in the absence of free Ca^{2+}_o following the addition of EGTA to the media (data not shown).

F. Other potential members of the CaSR to p62 pathway.

Although c-Src is frequently co-localized with numerous receptor tyrosine kinases, its physical presence in multi-protein complexes associated with signaling from 7TM receptors has not been reported until recently. Lefkowitz et. al. have characterized the activation of c-Src in response to stimulation of the lysophosphatidic acid (LPA) receptor and α-adrenergic receptor (α-AR) (Luttrell, Hawes et. al., 1996). Upon ligand binding to these receptors, Src is activated and becomes complexed with the adaptor protein Shc.

We reasoned that a similar mechanism might explain the interactions

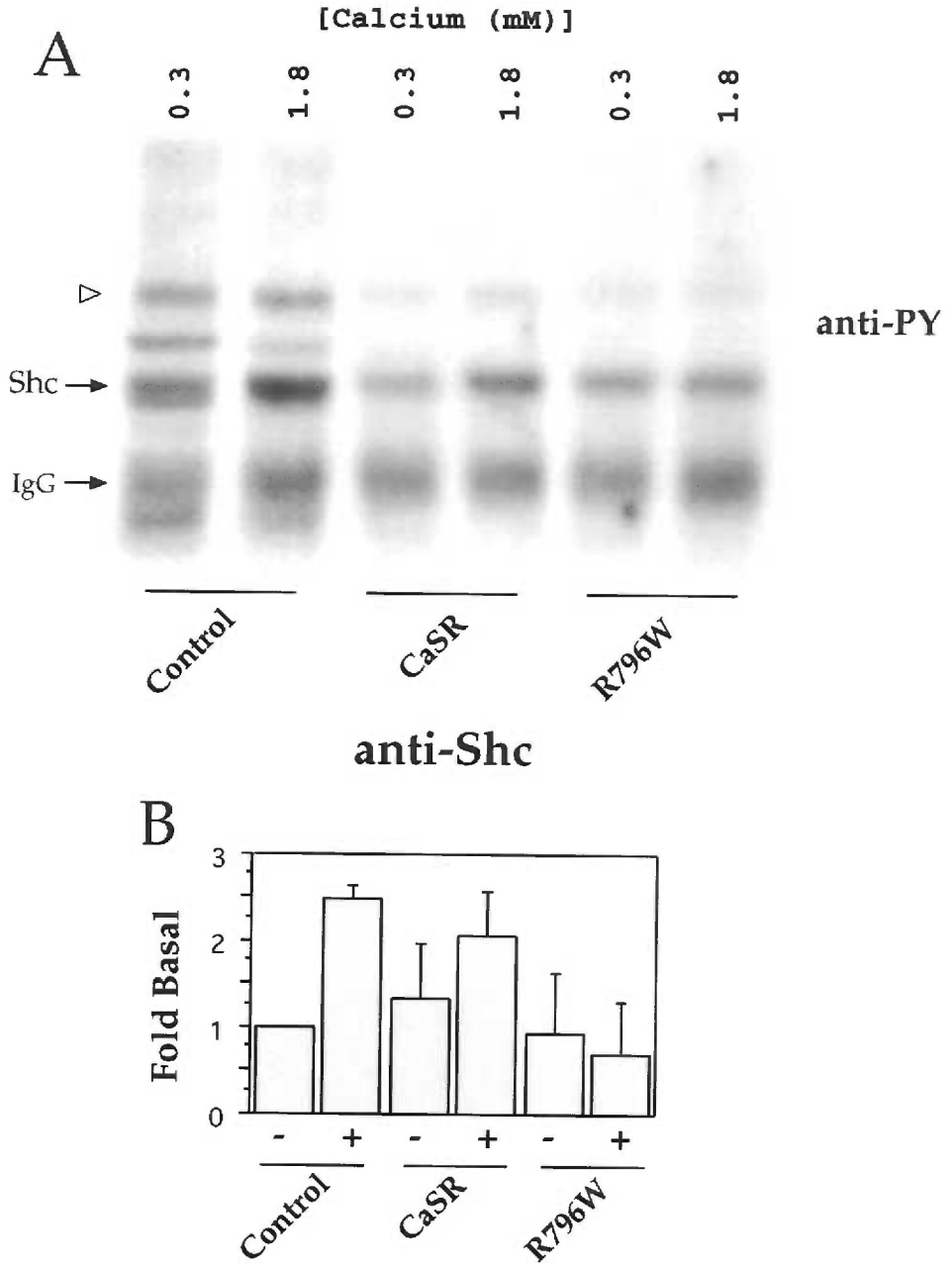

Figure 13. Effect of CaSR and R796W mutant on calcium-induced Shc phosphorylation. (A) Rat1 cells stably transfected with vector alone (Control), the CaSR, or R796W CaSR were grown to confluence in normal DMEM then cultured in low (0.3 mM) calcium-containing media for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Resulting lysates were immunoprecipitated with anti-Shc antibodies, subjected to SDS-PAGE and immunoblotted with anti-PY. (B) Data are presented as fold increase over nonstimulated control cells and represent the mean +/- S.D. for three independent experiments.

between the CaSR and Src kinase in Rat1 cells. That is, activation of the CaSR might lead to an increase in phosphorylation state of the adapter protein Shc and its association with other proteins. To address this notion, we stimulated the stably transfected cells with calcium and observed the effect on the tyrosine phosphorylation of Shc. Figure 13A shows that phosphorylation of a 56 kDa form of Shc was significantly increased ($p < .05$, n=3) following calcium treatment in both control and CaSR overexpressing cells. This increase was attenuated in cells expressing the R796W mutant ($p < .05$ compared to stimulated controls). Similar results were obtained in three replicate experiments and are represented graphically in figure 13, panel B.

An additional tyrosine phosphorylated protein of approximately 62 kDa was also present in the Shc immunocomplex (figure 13A, open arrow). This 62 kDa phosphoprotein was not immunoreactive with anti-Shc antibodies, indicating that it was not one of the other Shc isoforms (data not shown).

G. Interactions between GAP, p62 and Shc.

Given the size of this Shc-associated 62 kDa protein, we tested the possibility that this protein was GAP-associated p62. Since an antibody capable of detecting p62 on Western blots is not presently available, we immunoprecipitated lysates with either anti-GAP antibody or anti-p62 antibody, and immunoblotted with anti-Shc antibodies. As seen in figure 14, Shc was observed to be constitutively present in the anti-GAP immunocomplex for all of the stable lines under both low and high Ca^{2+}_o. Shc protein was also detected in anti-p62 immuno-complexes from the wild-type and CaSR overexpressing cells, but was clearly absent in immunoprecipitations form the R796W line. Thus, Shc can be complexed

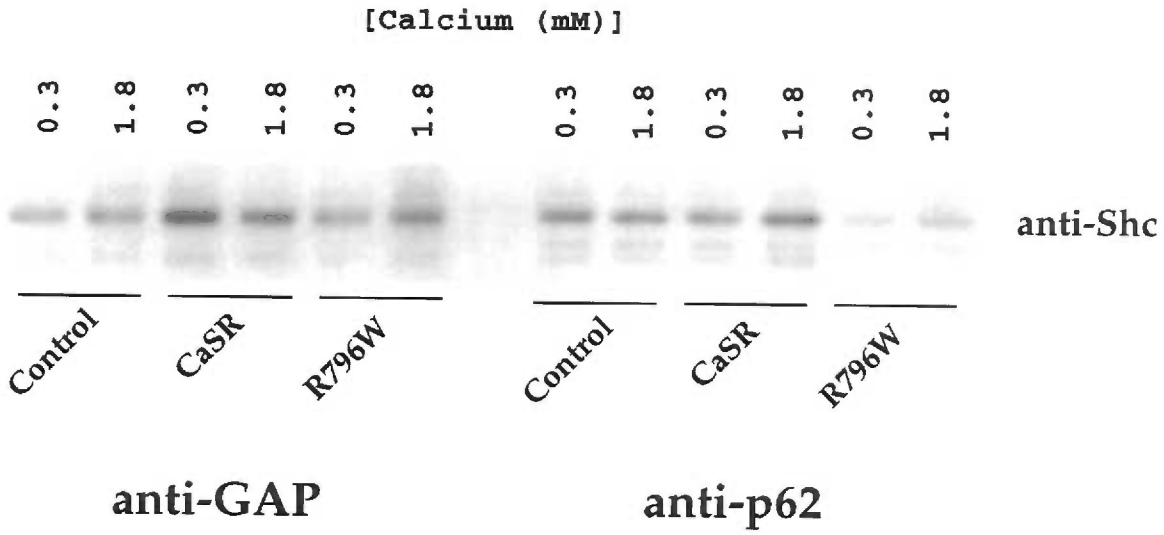

Figure 14. Shc association with GAP/p62 complex. Rat1 cells stably transfected with vector alone (Control), the CaSR, or the R796W mutant were grown to confluence in normal DMEM then cultured in low calcium-containing media for 4 hours. Cells were stimulated with 1.8 mM Ca^{2+} for 10 minutes and harvested in M-TG. Resulting lysates were immunoprecipitated with anti-GAP or anti-p62, subjected to SDS-PAGE and immunoblotted with anti-Shc.

with both GAP and p62 in Rat1 cells; however, the association between Shc and p62 appears to be disrupted in the presence of overexpressed R796W.

III. The CaSR and Proliferation in Rat1 Cells.

Data presented above suggest that the CaSR may modulate signaling events related to proliferation in Rat1 cells. Among these $Ca^{2+}{}_o$-sensitive events are the activation of Src kinase, the increased phosphorylation of Shc, and the amount of phosphorylated p62 present in the GAP immunocomplex. These observations led us to investigate the effects of overexpressing the CaSR on cell growth, using direct growth curves in cells stably transfected with vector, CaSR or R796W mutant. As seen in figure 15, overexpression of the CaSR resulted in a significant depression of the growth curve over a 72 hour period. Overexpression of the R796W CaSR, on the other hand, had no observable effect on the rate of cell growth compared to the control cells.

A second independently derived clone of CaSR-transfected Rat1 cells also showed decreased growth in comparison to control cells (data not shown). This independent confirmation suggests that the observed growth inhibition may not be attributable to integration artifacts. However, mechanisms responsible for the observed growth inhibition cannot be determined from these experiments.

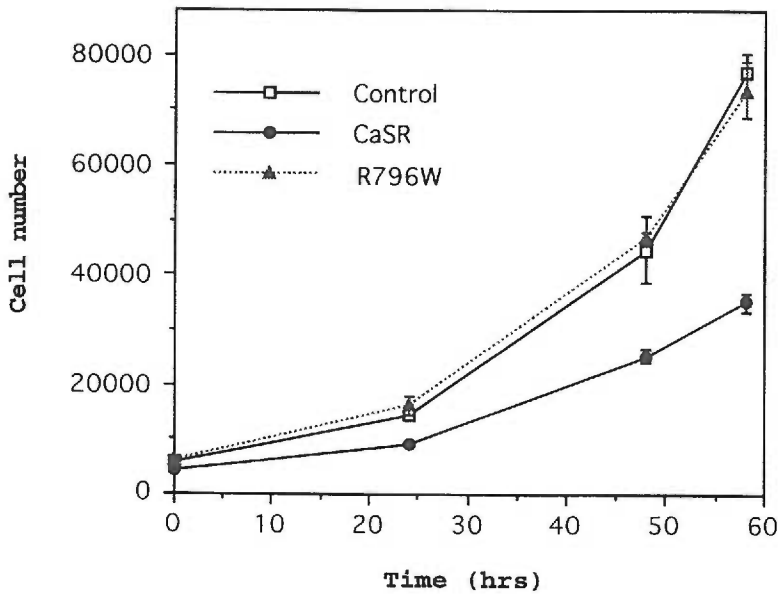

Figure 15. Growth curve of Rat1 cells stably transfected with CaSR or R796W mutant. Rat1 cells stably transfected with either vector alone (Control), CaSR, or R796W were seeded in 35 mm wells at low density and allowed to grow in normal DMEM (1.8mM Ca^{2+}) supplemented with 0.5% calf serum. Cells were harvested by trypsinization at the times indicated, and total cell number was determined by Coulter counter. Each data point represents the mean +/- S.D. of triplicate wells.

DISCUSSION AND CONCLUSIONS

Extracellular calcium plays a pivotal role in signaling pathways leading to growth and differentiation in a number of cell types. Studies in the keratinocyte model suggest that a proximal event in Ca^{2+}_o -mediated differentiation is tyrosine phosphorylation. An immediate increase in tyrosine phosphorylation is also observed in cultured fibroblasts when proliferation is stimulated by elevation of extracellular calcium. Specific cellular mechanisms through which this extracellular signal is transduced have not been fully elucidated.

The focus of this study is the potential interactions between the Calcium Sensing Receptor (CaSR) and pathways involving tyrosine phosphorylation in Rat1 fibroblasts. The effect of the CaSR on tyrosine phosphorylation in Rat1 cells was assessed following the stable transfection into these cells of expression vectors for the CaSR and the R796W inactivating mutant. In these cell lines, three different approaches all lead to the conclusion that the CaSR plays a major role in Ca^{2+}_o -mediated tyrosine phosphorylation.

First, we demonstrated that agonists of the CaSR are able to influence a number of signaling events involving tyrosine phosphorylation in Rat1 cells. We found that elevation of extracellular calcium was able to increase the level of Shc tyrosine phosphorylation and the association of tyrosine phosphorylated p62 with rasGAP. Similarly, the addition of the CaSR agonist gadolinium (Gd^{3+}) was able to induce c-Src kinase activity, as measured by autophosphorylation. The tyrosine phosphorylation state of proteins with molecular weights of approximately 44, 55, 63, 85, and 135kD was also influenced by stimulation with Ca^{2+} and/or Gd^{3+}.

Second, we present molecular evidence demonstrating expression of a CaSR in Rat1 cells. Using RT-PCR with primers directed against the CaSR, we identified a partial CaSR clone that was greater than 95% homologous with the reported rat cDNA. Additionally, we observed a 5.3kb transcript by northern blot analysis using the full length CaSR as a probe, comparable in size with the reported mRNA in rat and bovine tissues (Brown, Gamba et. al., 1993; Ruat, Molliver et. al., 1995). Immunoblot analysis using a polyclonal antibody raised against the CaSR also confirmed the presence of receptor protein in Rat1 cells.

Third, an inactivating mutant of the CaSR (R796W) was able to severely attenuate Ca^{2+}_o-induced signaling events involving tyrosine phosphorylation in Rat1 cells. Cells expressing the R796W mutant had decreased Gd^{3+}-stimulated c-Src kinase activity compared to wild-type and CaSR overexpressing cells. Attenuations in Ca^{2+}_o -stimulated tyrosine phosphorylation events were also observed as was inhibition of GAP/p62 complex formation and reduction in the amount of Shc present in the anti-p62 immunocomplex.

Taken as a whole, these data establish a role of the CaSR in Ca^{2+}_o-dependent pathways involving tyrosine phosphorylation in Rat1 fibroblasts. Our results provide a mechanism by which changes in extracellular calcium concentration, as sensed by the CaSR, can influence a number of signaling molecules implicated in cell growth and differentiation. Among these proteins are the Ras GTPase activating protein (GAP) and its associated protein p62, the adaptor protein Shc, and the Src cytoplasmic tyrosine kinase.

Interactions between the CaSR, GAP and p62.

In this study, we have found that the amount of tyrosine phosphorylated p62 present in anti-GAP immunoprecipitates is increased 3-5 fold following elevation of extracellular calcium. Increases the amount of tyrosine phosphorylated p62 associated with GAP have been reported in response to stimulation with a number of mitogens (Ellis, Moran et. al., 1990; Hosomi, Shii et. al., 1994; Kaplan, Morrison et. al., 1990; Medema, De Vries-Smits et. al., 1995; Moran, Polakis et. al., 1991). Increases in the amount of tyrosine phosphorylated p62 associated with GAP also appears to a proximal and specific event accompanying $Ca^{2+}{}_o$ -induced differentiation in keratinocytes (Filvaroff, Calautti et. al., 1992).

There are conflicting reports as to the mechanism of action regulating the formation of the GAP-p62 complex. Park and Jove have presented data suggesting that the interaction between GAP and p62 is dependent on tyrosine phosphorylation of GAP in v-Src transfected cells (Park and Jove, 1993). Tyr-457 of GAP is the major tyrosine residue phosphorylated by Src and RTK's (Liu and Pawson, 1991; Park, Liu et. al., 1992). Park et al reported a 3-fold reduction in the ability of p62 to bind to GAP which had mutations in this residue (Y457F) compared to wild-type GAP (Park and Jove, 1993). The GAP Y457F mutant was also defective in its ability to associate with the membrane fraction compared to wild-type GAP, an event which had previously been reported to involve the interaction of GAP with p62 (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991). The authors therefore concluded that phosphorylation of GAP plays an important role in GAP-p62 complex formation.

In contrast to this, Marengere and Pawson demonstrated that the interaction between p62 and GAP in v-Src transfected cells is dependent on

the N-terminal SH2 domain (SH2-N) of GAP (Marengere and Pawson, 1992). Their study was based on previous works that had shown that SH2-N could bind p62 *in vitro* (Koch, Moran et. al., 1992; Moran, Koch et. al., 1990). As a follow-up to this study, Marengere and Pawsons were able to demonstrate that mutations to this domain led to the complete loss of p62 binding from v-Src cell lysates (Marengere and Pawson, 1992). Given the high affinity of SH2 domains for phosphorylated tyrosine residues, it follows that the GAP-p62 complex formation would be facilitated by tyrosine phosphorylation of the p62 protein. This is in contrast to, though not necessarily incompatible with, the model presented by Park et al, where formation of the GAP-p62 complex is dependent upon phosphorylation of GAP (Park and Jove, 1993). Thus, mechanisms regulating the association of GAP with p62 are complex and may involve multiple SH2 interactions.

Given these conflicting reports, we attempted to address whether the increase in the amount of tyrosine phosphorylated p62 associated with GAP (and presumably GAP-p62 complex formation) observed in our study might be due to the phosphorylation of GAP or the phosphorylation of p62. To test the influence of GAP phosphorylation on the GAP-p62 complex, we immunoprecipitated lysates with anti-GAP and immunoblotted with anti-PY. We were unable to detect tyrosine phosphorylation of GAP with or without $Ca^{2+}{}_o$ addition, despite using up to 1mg of lysate for immunoprecipitation (data not shown). This result is not surprizing, given the absence of detectable GAP phosphorylation in non-transformed cells and the relatively minor increase in $Ca^{2+}{}_o$-induced tyrosine phosphorylation compared to treatment with other mitogens (Moran, Polakis et. al., 1991) and figure 4). However, we can not rule out the possibility that phosphorylation of GAP, albeit it beyond our ability to detect, contributes to the formation of the GAP-

p62 complex.

To test the influence of Ca^{2+}_o-stimulation on phosphorylation of p62, we conducted immunoprecipitation studies using the Roth antibody (2C4), which specifically recognizes GAP-associated p62 (Hosomi, Shii et. al., 1994; Neet and Hunter, 1995; Ogawa, Hosomi et. al., 1994). Surprisingly, we found that p62 was constitutively phosphorylated in the control Rat1 cells and in the stable transfectants. Immunoblotting with anti-GAP, however, revealed an apparent Ca^{2+}_o-sensitive increase in the amount of GAP associated with the p62 signaling complex. Futhermore, the association between GAP and p62 was disrupted in cells expressing the R796W mutation.

These results suggest two novel conclusions. The first is that the Ca^{2+}_o-induced increase in tyrosine phosphorylated p62 seen in anti-GAP immunoprecipitations is likely due to the increased formation of the GAP-p62 complex, as opposed to the phosphorylation of p62 in a pre-existing GAP-p62 complex. Secondly, it appears that phosphorylation of p62 is not sufficient for formation of this complex. This conclusion is supported by the observation that GAP-p62 complexes were not detected in cells expressing the mutant receptor even though these cells exhibited roughly the same amount of phosphorylated p62 in anti-p62 immunoprecipitations as the control cells.

Our data would at first seem to contradict the model proposed by Marengere et al in which formation of the p62-GAP complex is mediated by phosphorylation of p62 (Marengere and Pawson, 1992). This discrepancy may be due to the differences in the two paradigms utilized. The Marengere study was conducted in rat fibroblasts transfected with the v-Src oncogene. It is well documented that total cellular tyrosine phosphorylation is dramatically increased in the presence of this constitutive tyrosine kinase (Bouton, Kanner et. al., 1991; Brott, Decker et. al., 1991; Hamaguchi, Grandori et. al., 1988;

Kamps and Sefton, 1988; Kanner, Gilmer et. al., 1989; Pronk, Polakis et. al., 1992). Additionally, using wild-type Src and v-Src transfected NIH 3T3 cells, Neet et al have data demonstrating that the p62 protein detected by the Roth antibody is among these phosphoproteins heavily phosphorylated in the presence of v-Src (Neet and Hunter, 1995). It is quite possible, therefore, that the SH2 dependent GAP-p62 interaction reported by Marengere et al may not reflect normal SH2 interactions in non-transformed cells.

A second explanation for this difference may be due to the possibility that the GAP-associated protein observed with v-Src transfected cells in the Marengere report is not the p62 recognized by the Roth antibody used in our study. For example, a GAP-associated phosphoprotein doublet has been reported following $Ca^{2+}{}_o$-stimulation in fibroblasts and keratinocytes (Medema, De Vries-Smits et. al., 1995). The authors also suggested that the higher 65 kDa phosphoprotein was different from the lower p62, as the 65 kDa protein was not recognized by the Roth antibody (2C4). In addition, several groups have reported a phosphoprotein doublet of approximately 62kDa in v-Src transfected cells following anti-GAP immunoprecipitations (Bouton, Kanner et. al., 1991; Neet and Hunter, 1995; McNeil and Rodland, unpublished observations). Further characterization of the GAP-p62 interaction awaits cloning of the specific GAP-associated p62 protein(s).

Although the biological significance of the GAP-p62 complex is still unclear, there is increasing evidence that p62 may act as a docking protein that recruits signaling molecules to the membrane. Neet et. al. have reported that the complex between p62 and the tyrosine kinase CSK is preferentially localized to the membrane fraction (Neet and Hunter, 1995). Other groups have shown that GAP is found predominantly in the membrane fraction

when it is bound to p62 (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991). Upon activation, whether by phosphorylation or an as yet unknown mechanism, p62 would then mediate the translocation of GAP or other signaling molecules to membrane regions. In the case of the GAP-p62 complex, this translocation places GAP in close proximity to Ras, thereby enabling GAP to negatively regulate this molecular switch.

Activation of Shc by the CaSR

Another signaling molecule affected by changes in Ca^{2+}_o was the adapter protein Shc. As described above, Shc is an adapter protein which is involved in the activation of Ras by G-protein coupled receptors and receptor tyrosine kinases (RTK's) (Pelicci, Lanfrancone et. al., 1992; Pronk, McGlade et. al., 1993; Rozakis-Adcock, McGlade et. al., 1992). In this study, we report that the tyrosine phosphorylation state of an approximately 56 kDa form of Shc, presumed to be the reported $p52^{Shc}$ isoform, was increased 2.5 fold upon elevation of extracellular calcium.

The significance of Shc phosphorylation is most apparent in the ability of the protein to form signaling complexes with other SH2 containing proteins. Upon phosphorylation, Shc can associate with the adaptor protein Grb2 and the Ras guanine exchange factor (GEF), Sos. This interaction is dependent on tyrosine 317 in Shc's CH1 domain, as mutants lacking this residue can not bind to Grb2 (Salcini, McGlade et. al., 1994). Similarly, overexpression of this Tyr317 mutant in mouse fibroblasts does not induce neoplastic transformation as does overexpression of the wild-type protein (Pelicci, Lanfrancone et. al., 1992; Salcini, McGlade et. al., 1994). Futhermore, expression of this mutant in chinese hamster lung fibroblasts (CCL39 cells) exerts a dominant negative phenotype on Ras-dependent signaling events,

including MAP kinase activation, gene expression and cell growth (Chen, Grall et. al., 1996). Thus, Shc phosphorylation plays an important role in many signaling pathways.

Our data demonstrate a CaSR-dependent phosphorylation of Shc in Rat1 fibroblasts in response to elevated extracellular calcium. Since formation of a Shc-Grb2-Sos signaling complex has been shown to activate Ras, it is quite possible that Ca^{2+}_o -stimulated phosphorylation of Shc may potentially influence Ras via a similar mechanism (Pronk, Alida et. al., 1994). In support of this notion, Huang et al have reported the activation of MAP kinase in response to elevation of extracellular calcium in human fibroblasts (Huang, Maher et. al., 1995). The authors did not directly test the effect of Ca^{2+}_o on Ras activity, however. The prospect that activation of the CaSR may regulate Ras-dependent events warrants further investigation and is discussed below.

Although we found all three characterized isoforms of Shc to be present in Rat1 cells (data not shown), only the $p52^{Shc}$ isoform (~56 kDa in our study) exhibited an increase in tyrosine phosphorylation and was detectable in anti-GAP and anti-p62 immunoprecipitations in response to Ca^{2+}_o. Isozyme-specific increases in Shc tyrosine phosphorylation have also been reported in various cell types. Pronk et. al. observed an 1.8-fold increase in tyrosine phosphorylation of the $p52^{Shc}$ isoform upon insulin treatment in CHO cells (Pronk, McGlade et. al., 1993). A slight increase in phosphorylation of the $p46^{Shc}$ isoform was detected in these cells with prolonged exposure, but phosphorylation of the $p66^{Shc}$ isoform was not reported. In rat fibroblast, Pelicci and collegues reported an increase in EGF-stimulated tyrosine phosphorylation of $p46^{Shc}$ and $p52^{Shc}$, but markedly reduced tyrosine

phosphorylation in the $p66^{Shc}$ isoform (Pelicci, Lanfrancone et. al., 1992). Finally, Chen et al found that the $p52^{Shc}$ variant was the major phosphorylated isoform following stimulation of the G-protein coupled thrombin receptor in CCL39 cells (Chen, Grall et. al., 1996).

The differential phosphorylation of these isozymes may reflect the functional diversity of Shc. Mouse fibroblasts overexpressing the $p46^{Shc}$ or $p52^{Shc}$ isoforms acquire a transformed phenotype *in vitro* and form tumors when injected into athymic mice (Pelicci, Lanfrancone et. al., 1992). Unlike $p46^{Shc}$ and $p52^{Shc}$, however, overexpression of the $p66^{Shc}$ variant does not induce tumor formation in mouse fibroblasts (Bonfini, Migliaccio et. al., 1996). The authors attribute these differences to the presence of an additional carboxy-terminal domain in the $p66^{Shc}$ isoform (Bonfini, Migliaccio et. al., 1996). As noted, Shc $p66^{Shc}$ is a splicing variant of the the $p46/52^{Shc}$ isoforms with an additional glycine/proline rich region referred to as the CH2 domain. The $p66^{Shc}$ CH2 domain may therefore interact with a different set of SH3-containing proteins than the CH1 domain which is shared by all isoforms.

Not all of Shc's protein-protein interactions are dependent on tyrosine phosphorylation, however. Matoskova and collegues have presented evidence indicating that Shc's association with the Eps8 protein, a substrate of the EGF receptor, is mediated by the latter's SH3 domain (Matoskova, Wong et. al., 1995). In serum-starved NIH3T3 cells, Shc and Eps8 were able to associate under conditions where they display little, if any, tyrosine phosphorylation, thus arguing against a SH2- or phosphorylated tyrosine binding (PTB)-dependent association. In addition, a Eps8-SH3-GST fusion protein was capable of associating with Shc in a Far Western assay, indicating a SH3-mediated interaction.

These SH3-dependent interactions may help explain results obtained in

our study. Despite the Ca^{2+}_o-sensitive changes in Shc tyrosine phosphorylation, we found that Shc was constitutively present in anti-GAP and anti-p62 immunoprecipitations. It is conceivable, then, that the association between Shc and GAP/p62 is independent of Shc's tyrosine phosphorylation state.

These results should be interpreted with caution due to the possibility that techniques utilized in this study may not be optimized to adequately characterize phosphorylation-dependent interactions between Shc and GAP/p62. A more stringent assessment of these interactions might involve overexpression of wild-type and Tyr317 mutants of Shc and monitoring its association with GAP and p62 following Ca^{2+}_o treatment. Under these conditions, any change in the interactions between Shc and GAP/p62 could be correlated with Shc phosphorylation. Perturbations of this paradigm would also include overexpression of wild-type and Tyr457 mutants of GAP. Ca^{2+}_o-dependent interactions between Shc, GAP and p62 could be correlated with their respective phosphorylation state. Positive results could then be reproduced in cells overexpressing the CaSR and R796W mutant to determine the role of the CaSR on these Shc-GAP-p62 interactions.

Regardless of the mechanism of interaction, our finding that Shc associates with GAP and p62 has significant implications. The association of Shc with the Grb2-Sos complex results in the activation of Ras (Pronk, Alida et. al., 1994). In this study, we demonstrate the constitutive association between Shc and the Ras GTPase, p120/GAP. We also report that the amount of GAP present in anti-p62 immunoprecipitations is increased upon activation of the CaSR. Given the influence of GAP and Shc on Ras activity, it is conceivable that activation of the CaSR may contribute to the regulation

of Ras. In Rat1 cells, it appears that a common constituent of these Ras-related signaling complexes is p62.

p62 is thought to act as a docking protein for GAP and other phosphoproteins to enable their translocation from the cytosol to the membrane (Molloy, Bottaro et. al., 1989; Moran, Polakis et. al., 1991; Neet and Hunter, 1995). It follows then, that one of the effects of calcium stimulation may be the placement of GAP in close proximity to Ras, thereby inactivating Ras. Consistent with this hypothesis, Medema and co-workers have reported that EGF-induced activation of Ras and MAP kinase is inhibited in human keratinocytes following pretreatment with 1.8 mM calcium (Medema, Sark et. al., 1994). The ability of the EGF receptor to autophosphorylate was not affected, indicating that extracellular calcium does not exert its influence on Ras at the level of the receptor itself. Although the authors did not directly assertain the role of GAP in these experiments, the increased association of phosphorylated p62 with GAP in response to elevated Ca^{2+}_o is well documented in keratinocytes (Filvaroff, Calautti et. al., 1992; Filvaroff, Calautti et. al., 1994).

These reports, combined with our data, suggest a model where both positive and negative regulators of Ras (ie Sos and GAP) might be found in the same signaling complex. The characterization of this model (ie the p62-Shc-GAP-Sos complex) may shed light on the overall regulation of Ras activity. Several reports have suggested that the *duration* of Ras activation, with the subsequent activation of MAP kinase, is the determining factor in shared pathways leading to cell growth or differentiation (Hill and Treisman, 1995; Marshall, 1995 *and references therein*). Assuming that the GAP-Shc interaction can be applied to other systems, it is conceivable that the duration of Ras activity might be attributed to the dynamics of the p62-Shc-GAP-Sos

complex. Indeed, the duration of Shc phosphorylation is highly dependent on the means of stimulation. For example, stimulation of PC12 cells with carbachol results in the transient (less than 1 minute) phosphorylation of Shc (Lev, Moreno et. al., 1995). Stimulation by this agonist is generally associated with acute cellular events such as membrane depolarization. NGF stimulation, on the other hand, results in the persistent phosphorylation of Shc for greater than 5 hours (Stephens, Loeb et. al., 1994). PC12 cells treated with NGF undergo Ras-dependent terminal differentiation, as monitored by neurite outgrowth. Thus, the duration of Shc phosphorylation correlates well with acute and longer term cellular events.

It is possible that the duration of the Ras signal may be regulated by the p62-Shc-Gap-Sos complex. As an initial test of this hypothesis, one could stimulate cultured cells and characterize the dynamics of the membrane-associated complex as a function of time. Any observed differences in tyrosine phosphorylation, protein localization or in protein-protein interactions, for instance, would then be correlated with Ras activity - as measure by Ras GTP charging or MAP kinase activation. Positive results could then be pursued by gain of function/loss of function studies using the components of the p62-Shc-Gap-Sos complex and monitoring the duration of Ras activity.

Potential role of cytoplasmic tyrosine kinases in CaSR-mediated pathways.

Data presented in this thesis implicate the CaSR in Ca^{2+}_o-dependent pathways involving changes in tyrosine phosphorylation. Elevation of extracellular calcium led to increased the levels of Shc phosphorylation in the presence of wild-type, but not mutant, CaSR. Additionally, the tyrosine phosphorylation state of unidentified proteins of approximately 44, 55, 63, 85,

and 135kDa was also influenced by treatment with extracellular Ca^{2+} and/or Gd^{3+} . It follows then, that these changes in tyrosine phosphorylation are likely due to the activation of a tyrosine kinase, or the inactivation of a protein tyrosine phosphatase (PTP).

Our initial experiments with herbimycin A (figure 4) argued for the activation of a kinase rather than inactivation of a PTP. We therefore looked at the effect of CaSR activation on a tyrosine kinase known to be sensitive to elevated Ca^{2+}_o, c-Src. We found that c-Src kinase activity was significantly increased ($p < .05$) when Rat1 cells were treated with Gd^{3+}. In addition, this increased phosphorylation was not observed in cells expressing the R796W mutant. Gd^{3+} is a specific agonist for the CaSR at the dosage used in this study (Brown, Gamba et. al., 1993; Riccardi, Park et. al., 1995). These data indicate that activation of the CaSR is sufficient for the induction of Src kinase activity. Similarly, the absence of kinase activity in the R796W transfected cells suggests that a functional CaSR is required for Gd^{3+}-stimulated Src kinase activation. It follows then, that activation of the CaSR by other agonists such as elevation of extracellular calcium, may also induce Src kinase activity. Taken together, these data suggest that activation of the CaSR in response to elevated Ca^{2+}_o leads to activation of Src kinase and subsequent substrate phosphorylation.

The induction of Src kinase activity in response to elevated Ca^{2+}_o might explain the results observed in our study with GAP and p62. Although the specific substrates of Src kinase are not totally defined, several groups have demonstrated that an increase in the amount of tyrosine phosphorylated p62 associated with GAP occurs in response to activation of Src (Bouton, Kanner et. al., 1991; Chang, Wilson et. al., 1993; Ellis, Moran et. al., 1990; Neet and Hunter, 1995; Park and Jove, 1993). Futhermore, we have

observed that both c-Src kinase activity and the GAP/p62 interaction are stimulated by agonists of the CaSR and are adversely affected by the R796W mutant. This correlation suggests that the CaSR may exert its influence on GAP-p62 complex formation by modulating Src kinase activity.

The effect of Ca^{2+}_o-stimulated Src activity is likely not limited to GAP/p62- related events. Recent work by Lefkowitz and collegues suggest that Src may also have a role in 7TM receptor-mediated Shc phosphorylation and activation of MAP kinase (Luttrell, Hawes et. al., 1996). The authors report that stimulation of the LPA receptor resulted in the concommitant activation of Src kinase and phosphorylation of Shc. In addition, Src protein was found to physically associate with Shc following LPA treatment. It should be noted that the temporal order of the Shc-Src interaction, whether phosphorylation of Shc induces Src activity or vice versa, is still unclear.

LPA-mediated MAP kinase activation can be inhibited by tyrosine kinase inhibitors (Hordijk, Verlaan et. al., 1994). The authors therefore questioned whether c-Src might be the kinase responsible for activation of MAP kinase following LPA stimulation. To address this, they overexpressed Src and observed the effect on MAP kinase activity. They found that overexpression of Src was able to mimic LPA treatment in the activation of MAP kinase and the increase in Shc phosphorylation (Luttrell, Hawes et. al., 1996). Additionally, overexpression of Csk kinase, an inactivator of Src, was able to abolish LPA-stimulated MAP kinase activity and Shc tyrosine phosphorylation. Taken together, these data implicate Src in LPA-mediated activation of MAP kinase and in the phosphorylation of Shc.

Although it remains to be seen whether the model presented by Lefkowitz can be ubiquitously applied to other 7TM systems, the similarities between his observations and ours warrant further investigation. A similar

Src-dependent mechanism, for example, may help explain results observed in our study. As an initial test of this hypothesis, Rat1 cells overexpressing Csk kinase could be stimulated with agonists of the CaSR while monitoring the effect on Shc phosphorylation. A significant decrease in Ca^{2+}_o-induced Shc phophorylation in cells transfected with Csk kinase compared to wild-type cells would support the application of the Lefkowitz model to our system.

It should be noted, however, that Src is certainly not the only tyrosine kinase which might participate in signaling pathways activated by the CaSR. Activation of the recently decribed Pyk2 tyrosine kinase, for instance, has been shown to be highly dependent on intracellular calcium levels (Lev, Moreno et. al., 1995). Furthermore, overexpression of Pyk2 results in a dramatic increase in the phosphorylation state of Shc. No direct association between Pyk2 and Shc has yet been demonstrated.

In a subsequent study, Dikic et. al. reported that Pyk2 was required for LPA-stimulated activation of Src in PC12 cells (Dikic, Tokiwa et. al., 1996). In addition, dominant negative forms of Pyk2 were able to inhibit MAP kinase activation by LPA. Since treatment with LPA has been documented to result in the association of Src with Shc in COS-7 cells, it is possible that Pyk2 in association with Src and Shc may provide a link between G-protein coupled receptors and MAP kinase activation. (Luttrell, Hawes et. al., 1996).

Since activation of the CaSR leads to IP_3 and Ca^{2+}_i release, activation of Pyk2 in our system is likely. However, we found that the ionophore A23187 did not mimic the Ca^{2+}_o-stimulated increase in the amount of tyrosine phosphorylated p62 associated with GAP (figure 4). Given the sensitivity of Pyk2 to levels of intracellular calcium, Pyk2 activation would be expected under these conditions. This argues against activation of Pyk2 as being

sufficient for the Ca^{2+}_o-stimulated increase in amount of tyrosine phosphorylated p62 associated with GAP. However, a role of Pyk2 in Ca^{2+}_o-stimulated pathways cannot be ruled out based on our experiments.

Potential Role of the CaSR in Proliferation and Differentiation

Extracellular calcium has been shown to play an important role in pathways leading to cell growth and differentiation (Hennings, Holbrook et. al., 1983; Hennings, Michael et. al., 1980; Huang, Maher et. al., 1995; Ochieng, Tahin et. al., 1991). Relevant to this study, Huang et al have demonstrated that elevation of extracellular calcium stimulates proliferation in human fibroblasts (Huang, Maher et. al., 1995). Conversely, human fibroblasts fail to replicate when the $[Ca^{2+}]_o$ is reduced from 1mM to 0.1mM (Morgan, Yang et. al., 1991). Data presented in our report suggest that the CaSR may be involved in transducing this Ca^{2+}_o-stimulated proliferative signal.

We have demonstrated that activation of the CaSR leads to changes in the tyrosine phosphorylation or kinase activity of several signaling proteins known to be associated with proliferation. For instance, elevation of extracellular calcium leads to the phosphorylation of Shc and increases the amount of phosphorylated p62 present in the GAP immunocomplex. Similarly, stimulation of the CaSR by Gd^{3+} results in the activation of Src kinase, as monitored by autophosphorylation. As noted, the Ca^{2+}_o-stimulated increases were diminished in the cells expressing the R796W mutant, indicating that these cellular events are sensitive to CaSR activation.

To better assess the role of CaSR agonists on proliferation, we tested the effect of polyvalent cations on ^{3}H-thymidine incorporation in fibroblasts and keratinocytes. We observed a dose dependent increase in ^{3}H-thymidine incorporation in human fibroblasts following stimulation with either

calcium or magnesium. These divalent ions are known to be activators of the CaSR within the dosages that were used in our study (Riccardi, Park et. al., 1995; Ruat, Snowman et. al., 1996). These two agents, and the polyvalent cation polylysine, were also capable of inhibiting ^{3}H-thymidine incorporation in keratinocytes, again at doses known to activate the CaSR. Taken together, these data suggest that activation of the CaSR may be involved in pathways leading to either cell growth or differentiation.

One limitation of our data involving ^{3}H-thymidine incorporation, however, is that the paradigm does not monitor cell growth directly. Instead, it is used to measure the amount of thymidine incorporated into DNA during synthesis, thus proliferation is inferred. Additionally, other parameters such as uptake of the nucleotide or nucleotide pool size may be affected in the presence of the agonist.

To alleviate some of these problems, proliferation could be monitored directly by growth curves. Fibroblasts and/or keratinocytes could be cultured in low calcium containing media, and then stimulated with increasing concentrations of calcium or other CaSR agonist. Growth rates could then be correlated with the agonist's reported effective dose for the CaSR. A similar pharmacological approach was successfully used by Brown et. al. to characterize the role of the CaSR in systemic calcium homeostasis (Brown, 1991; Brown, Gamba et. al., 1993).

It should be noted, however, that a more thorough paradigm to test the influence of the CaSR on Ca^{2+}_{o}-stimulated proliferation should also include gain of function/loss of function studies. Although we did not directly assess the role of the CaSR on Ca^{2+}_{o}-stimulated proliferation, we did find that overexpression of the wild-type receptor is growth inhibitory when cells were cultured in high (1.8 mM) calcium-containing medium. This inhibition was

observed with two independent clonal isolates and also when the CaSR was stably transfected in a rat ovarian cell line (data not shown). Overexpression of the R796W mutant receptor in Rat1 cells, conversely, had no observable effect on the rate of proliferation compared to the control transfected cells.

One explanation for these results is that expression of the CaSR may be tightly regulated by the cells. For reasons not yet understood, overexpression of the CaSR may be deleterious to normal signaling pathways with the end result being an inhibition of cell growth. Under this model, isolated stable transfectants would have been preferentially selected based on their relatively low level of CaSR expression. This notion is consistent with our growth data, and may help explain the lack of observable increases in tyrosine phosphorylation or kinase activity experiments in which the CaSR was overexpressed.

In support of this hypothesis, Brown and collegues have presented evidence that expression of the CaSR message changes over time in culture (Brown, Zhong et. al., 1995; Mithal, Kifor et. al., 1995). Primary cultures of bovine parathyroid cells are known to quickly (within 6 days) lose their responsiveness to extracellular calcium, as monitored by inhibition of PTH secretion. Using PTH secretion as an assay, Brown and collegues were able to correlate this loss of calcium responsiveness with a decrease in CaSR mRNA expression and, presumably, CaSR protein expression (Mithal, Kifor et. al., 1995). It is conceivable, then, that cells expressing lower amounts of the CaSR might have a proliferative advantage over their counterparts. This, in turn, would allow the low CaSR expressors to proliferate at a more rapid rate - thereby dominating the population and accounting for the reduction in CaSR message.

One limitation to our growth experiment is that it does not adequately

address the possible role of the CaSR as a mediator of the Ca^{2+}_o-stimulated proliferation signal. The above experiment was conducted at a set level of extracellular calcium, any conclusions made are therefore limited to the effect of the *presence* of the CaSR as opposed to *activation* of the receptor as a funtion of Ca^{2+}_o-stimulation. Cellular events arising from these two scenarios are not necessarily equivalent.

A preferred approach to addressing the role of the CaSR on proliferation might, therefore, include manipulating the level of CaSR expression in the presence of increasing concentrations of a CaSR agonist. Such a paradigm could be accomodated by stably expressing the receptor with an inducible promoter system. Cell growth could then be monitored against a dose curve of calcium or gadolinium - at known levels of exogenous receptor. Preferably, these experiments could be conducted in a cell line with a functionally null CaSR background such as chinese hamster ovary (CHO) cells. CHO cells have previously been used to study the effect of the CaSR on other signaling events including IP_3 production and release of arachidonic acid (Ruat, Snowman et. al., 1996).

Disruption of signaling pathways by the R796W mutant.

The ability of the R796W mutant to disrupt Ca^{2+}_o -mediated signaling was quite pronounced in several of the assays tested. For instance, Ca^{2+}_o-stimulated phosphorylation of Shc, Gd^{3+} -stimulated Src kinase activity, and the amount of phosphorylated p62 present in GAP immunocomplex following elevation of Ca^{2+}_o were all decreased in cells expressing the mutant receptor. The specific mechanism responsible for this inhibition is unclear, however.

One possible explanation for these results is that the mutation might

disrupt the normal protein conformation of the receptor in the third intracellular loop. This alternate configuration might then interfere with the coupling of the receptor to its intracellular effectors. The extracellular signal normally transduced by the CaSR, would then be disrupted in cells expressing the mutant receptor. In support of this notion, Pollak has suggested that the lack of a "critical" number of functional receptors might explain the phenotype exhibited in patients with familial hypocalciuric hypercalcemia (FHH) (Pollak, Brown et. al., 1993).

Such a scenario is certainly consistent with studies in oocytes characterizing the R796W mutation as "inactivating" (Bai, Quinn et. al., 1996; Pollak, Chou et. al., 1994). However, this model does not adequately account for the inhibition of Ca^{2+}_o-sensitive events reported here. For example, we demonstrate the inhibition of protein-protein interactions in the mutant cells *prior* to Ca^{2+}_o stimulation. Compared to wild-type cells and cells overexpressing the CaSR, cells expressing the R796W mutant displayed severely disrupted protein-protein interactions involving p62 both basally and following calcium stimulation. Several proteins were also observed to have increased basal tyrosine phosphorylation in lysates isolated from the cells expressing the inactivating mutant.

Our findings regarding the absence of the GAP-p62 complex in the cell line expressing the R796W mutant do not take into account the possibility that the level of the p62 protein has been altered in this particular line. This is partially due to the unavailability of a blotting antibody capable of detecting the p62 protein. An alternative method to monitor relative protein levels would be to quantitate the 62kD protein from cells labelled with ^{35}S-methionine and then immunoprecipitate with the Roth antibody. One could then monitor relative levels of the 62kD protein by band intensity (dpm).

However, the presence of additional 55-62 kDa proteins observed in the p62 immunoprecipitation (figure 10) are likely to interfere with a clear interpretation. To remedy this, the non-specific binding of the antibody might be reduced by boiling the lysate in SDS prior to immunoprecipitation. However, the ability of SH2 signaling complexes to be reconstituted following this procedure may also lessen the chances for an unambigous result (Neet and Hunter, 1995).

It should be noted, however, that the R796W mutant did not alter the levels of expression of other tyrosine phosphorylated proteins in this study. For example, the relative protein amounts of GAP, Src and Shc were similar in wild-type Rat1's and cells transfected with the CaSR and R796W mutant (figures 9, 11 and data not shown). It seems likely therefore, that results observed with p62 in this study are not a general artifact of changes in protein levels. However, specific effects of the R796W mutant on p62 protein levels can not be ruled out.

Disruption of normal CaSR function in cells overexpressing R796W may reflect a direct interaction between wild-type and mutant CaSR, such that the mutant receptor actively interferes with the function of the endogenous CaSR. Such an interpretation is reminiscent of what is reported in many studies using "dominant negative" mutations. Dominant negatives have the ability to greatly attenuate an activity observed for the wild-type protein. Transduction pathways relying on the protein of interest can therefore be severely disrupted in the presence of its dominant negative. For this reason they are a very useful tool in characterizing the role of the wild-type protein in signaling pathways.

Two of the best studied examples of this are seen in dominant negative

forms of the EGF receptor (EGFR) and p21/Ras. Kinase-inactive mutants of the EGFR are able to suppress signaling when co-expressed with the wild-type receptor (reviewed in van der Geer, Hunter et. al., 1994)). This is due to the requirement for receptor dimerization and subsequent transphosphorylation for full kinase activity and substrate phosphorylation. Since the kinase-deficient molecule can not phosphorylate its partner, the net effect is a disruption of the transducing signal.

The ability of the CaSR R796W mutant to disrupt signaling by a similar mechanism would require intramolecular crosstalk between receptors. Such a scenario is not entirely without precedence, even in 7Tm receptors. In an elaborate set of experiments, Wess and collegues have presented evidence that inactive, chimeric mutants of the adrenergic and muscarinic receptors are able to compensate for each other's deficiencies when cotransfected into COS-7 cells (Maggio, Vogel et. al., 1993). Although no direct evidence was presented, the authors suggested that this phenomenon was due to formation of receptor dimers. It is interesting to note that in one particular experiment, portions of the third intracellular loop were involved in the intramolecular exchange, - as is the site of the R796W mutation in the CaSR. It is possible, although quite speculative, that the R796W mutation disrupts some dimerization-sensitive signaling event.

Another potential mechanism of action for the R796W mutant may be in a manner similar to the dominant negative forms of Ras. Two mutants of Ras are known to interfere with endogenous forms of the protein (reviewed in Bollag and McCormick, 1991). One dominant mutant functions by sequestering downstream effectors, thereby negating pathways normally utilized by wild-type Ras (Gibbs, Schaber et. al., 1989; Michaeli, Field et. al., 1989). The second mutation derives its interfering property by sequestering

Sos, thereby inhibiting normal Ras activation (Feig and Cooper, 1988; Powers, O'Neill et. al., 1989).

Classifying the R796W mutant as a Ras-like dominant negative would imply that the physical availability of 7TM receptors' intracellular effectors is rate limiting. The primary effectors of 7TM receptors, G-proteins, are generally believed to be in molar excess compared to the receptors. This argues against a mechanism where G-proteins are sequestered by the R796W mutant. However, 7TM receptors also utilize other intracellular effectors for transducing the extracellular signal. For example, in several 7TM receptors the process of desensitization is due to the agonist-stimulated phosphorylation of their intracellular loops and subsequent interaction with non-G protein effectors such as arrestin (reviewed in Hausdorff, Caron et. al., 1990; Lefkowitz, 1993). It may also be relevant to note that the R796W mutation lies within a putative PKC phosphorylation site in the third intracellular loop of the receptor (Pollak, Brown et. al., 1993). It is possible, then, that disruption of this site may result in the preferential coupling of an effector to the mutant receptor. Analogous to the Ras dominant negative, the sequestration of this molecule by the R796W mutant would have an adverse effect on signaling normally accomodated by the wild-type receptor.

One approach to test this hypothesis would be to obtain an antibody capable of immunoprecipitating the CaSR. An antibody raised against the extracellular domain of the receptor would allow the antibody to recognize both the wild-type receptor and the R796W mutant. Cell lines overexpressing the CaSR or R796W mutant could then be metabolically labeled with ^{35}S-methionine and immunoprecipitated with this antibody. CaSR-associated proteins which were differentially observed between the two cell lines could then be isolated and characterized.

Physiologic applications of this study

An obvious physiologic application to this study may be in the mechanism of PTH secretion in parathyroid cells. An inverse relationship exists between serum calcium levels and PTH release in parathyroid cells (reviewed in Brown, Pollak et. al., 1995b; Brown, Vassilev et. al., 1995; Brown, 1991). While the CaSR is directly implicated in this event, specific intracellular mechanisms mediating this release are not known.

The addition of high calcium to cultured parathyroid cells has long been associated with an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Shoback, Thatcher et. al., 1983). Activation of the CaSR by high calcium and other polyvalent cations results in an IP_3-mediated release of calcium from intracellular stores (Brown, Gamba et. al., 1993; Riccardi, Park et. al., 1995; Ruat, Snowman et. al., 1996). Increases in $Ca^{2+}{}_i$, however, do not appear to correlate with the associated changes in PTH release (reviewed in Brown, 1991). For example, the addition of ionomycin to parathyroid cells cultured in low calcium (0.5 mM) fails to produce an inhibition of PTH release, despite raising cytoplasmic calcium levels twofold (Nemeth, Wallace et. al., 1986). Furthermore, polyvalent cations which are known to activate the CaSR are able to produce a maximal inhibition of PTH release in these cells, but with considerable smaller increases in $Ca^{2+}{}_i$ than are observed with elevation of $Ca^{2+}{}_o$ (Brown, Chen et. al., 1989; Chen, Anast et. al., 1987; Nemeth and Scarpa, 1986; Shoback, Membreno et. al., 1988; Wallace and Scarpa, 1982). These observations therefore argue against $Ca^{2+}{}_i$ as being the sole regulator of PTH secretion.

In this study, we demonstrate that the CaSR is a transducer of $Ca^{2+}{}_o$-stimulated tyrosine phosphorylation. It is tempting to speculate that the

influence of the CaSR on tyrosine phosphorylation reported in this study may be applicable to the parathyroid system. The activation of various other 7TM receptors results in the induction of tyrosine phosphorylation in a number of other cell types. In cells expressing the α-adrenergic receptor, for instance, the epinephrine-induced phosphorylation of Shc correlates well with activation of MAP kinase and the onset of proliferation (Luttrell, Hawes et. al., 1996; van Biesen, Hawes et. al., 1995 *and references therein*). It is conceivable that activation of the CaSR in parathyroid cells may also stimulate the tyrosine phosphorylation of Shc and other signaling molecules. A pathway analagous to that utilized by the α-adrenergic receptor , albeit negative regulating, might influence PTH secretion in parathyroid cells.

Testing this hypothesis in the parathyroid system would be fairly straightforward. PTH release in these cells in response to CaSR agonists could be tested for its sensitivity to tyrosine kinase inhibitors such as genestein and herbimycin. Positive results would then allow for more elaborate paradigms described in this study, such as kinase assays and Western blotting. Any observed changes in Shc phosphorylation could then be more fully characterized by methods used by the Lefkowitz group to identify Shc and Src as downstream effectors of the α-adrenergic receptor (Luttrell, Hawes et. al., 1996). For example, one could introduce the dominant negative form of Shc or the βγ-subunit of the respective G-protein (presumably G_i) to parathyroid cells and observe the effect on Ca^{2+}_o-dependent PTH secretion.

An additional physiological application of this study may be in the area of wound healing. Three types of skin cells are implicated in this event, fibroblasts, keratinocytes and melanocytes (reviewed in Langdon, 1990). Within 24 hours of a laceration or abrasion to the skin there is a migration

and proliferation of fibroblasts into the affected area. The fibroblasts then proceed to secrete a protective layer of extracellular matrix, whose components include a combination of collagens and heparins. Eventually the keratinocytes will proliferate to fill the wound then differentiate to produce layers of keratin, forming a barrier against the external environment. The role of melanocytes is one of exclusion from the site - the discoloration of scar tissue reflects their inability to migrate into the affected area. The orchestrated balance in proliferation between these cell types, then, is a contributing factor to the wound healing process.

The differences in the growth rates of these cell types at various times after wounding have classically been attributed to their selective responsiveness to growth factors such as PDGF and the TGF's α and β. It is quite possible that one of the initiating signals in wound healing may involve changes in levels of extracellular calcium. Hennings et al have shown that $[Ca^{2+}]_o$ is higher in the non-proliferating cornified layers of the epidermis than in the basal layers where keratinocytes are undergoing active proliferation (Hennings, Holbrook et. al., 1983). Conversely, Bos and colleagues have demonstrated that fibroblasts proliferate more readily under conditions of high calcium (Huang, Maher et. al., 1995). A laceration to the epidermis, then, might expose the tissue to serum levels of calcium and favor the growth of fibroblasts over keratinocytes. As the area is sealed with coagulants, ECM, and other blood proteins, levels of extracellular calcium might then be reduced. This, in turn, would favor the proliferation of keratinocytes over fibroblasts.

The intrinsic ability of these cell types to respond to changes in extracellular calcium requires an appropriate Ca^{2+}-sensing mechanism. The demonstrated ability of the CaSR to transduce the Ca^{2+}-mediated signal and

influence proteins implicated in proliferation supports a role for the CaSR in the wound healing process. In this study we have reported the presence of a functional CaSR in Rat1 fibroblasts. It is interesting to note that a partial CaSR cDNA has also been identified in human keratinocytes. Although the gene program mediated by changes in Ca^{2+}_o is opposite in these cell types, i.e. one of proliferation in fibroblasts versus differentiation in keratinocytes, the CaSR is a strong candidate for transducing both types of responses to changes in Ca^{2+}_o.

In support of this notion, data presented in this study suggests that agonists of the CaSR are capable of inducing proliferation or differentiation, depending on the cell type. Quiescent human fibroblast had increased ^{3}H-thymidine incorporation when stimulated with either calcium or magnesium (figure 1). Conversely, proliferating human keratinocytes exhibited decreased ^{3}H-thymidine incorporation when treated with calcium, magnesium or polylysine (figure 2). All of these polyvalent cations are capable of stimulating the CaSR within the dosages used in this study (Ruat, Snowman et. al., 1996). Taken together, these results suggest that activation of the CaSR may play a part in the wound healing process.

SUMMARY

In this study, we showed that Rat1 fibroblast express a functional calcium-sensing receptor (CaSR). Activation of the CaSR by Ca^{2+}_o and/or Gd^{3+} influenced a number of growth- and differentiation-related events involving tyrosine phosphorylation. Included among these processes was the activation of Src kinase, the increased association of tyrosine phosphorylated p62 with GAP, and the increased tyrosine phosphorylation of Shc. All of

these signaling events were disrupted in the presence of a non-functional mutant of the CaSR. Additionally, we present data suggesting that the addition of CaSR agonists such as calcium, magnesium and polylysine to cultured cells was sufficient to induce proliferation, in the case of fibroblasts, or growth arrest in keratinocytes. This strengthens evidence supporting calcium interations with the CaSR, rather than calcium influx, as the signal mediating proliferation-associated responses.

These results confirm our hypothesis that the CaSR is a transducer of Ca^{2+}_o-stimulated tyrosine phosphorylation in Rat1 cells. Our results provide a mechanism by which changes in Ca^{2+}_o concentration, as sensed by the CaSR, can influence growth- and differentiation-related signaling pathways .

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